Diversity in natural product families is governed by more than enzyme promiscuity alone: establishing control of the pacidamycin portfolio

Sabine Grüschow,[a] Emma J. Rackham[a] and Rebecca J. M. Goss*[a]

[a] *School of Chemistry, University of East Anglia, Norwich, UK. Fax: 44 (0)1603 592-003; Tel 44 (0)1603 593-766; E-mail: r.goss@uea.ac.uk*

Experimental Section

Materials and bacterial strains. Microbiological media, buffer components, and reagents were purchased from BD Biosciences (Oxford, UK), Melford (Chelsworth, UK), Sigma-Aldrich (Haverhill, UK), Alfa Aesar (Hyesham, UK) and used without further purification. *Pfu* DNA polymerase was purchased from Promega (Southampton, UK), restriction enzymes were obtained from Roche Diagnostics (Burgess Hill, UK). *Streptomyces coeruleorubidus* AB1183F-64 was obtained from the Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research (NRRL, Peoria, USA). *Streptomyces lividans* TK24 and *Streptomyces coelicolor* M1154 was provided by Prof. Mervyn J. Bibb (John Innes Centre, Colney, Norwich, UK). *Escherichia coli* DH10B-T1 (Invitrogen, Paisley, UK) was used for routine cloning, *E. coli* BL21(DE3) (Novagen, Merck Biosciences, Nottingham, UK) was used for protein expression.

General DNA manipulations. Routine microbiological procedures were carried out according to standard protocols.[1,2] Restriction enzymes, ligase and *Pfu* DNA polymerase were obtained from Roche Diagnostics Ltd. (Burgess Hill, UK) or Promega (Southampton, UK) and used according to the manufacturer's instructions. Routine DNA sequencing was carried out at the University of Cambridge DNA Sequencing Facility using an Applied Biosystems 3730xl DNA Analyser.

Gene disruptions. Gene disruptions were carried out by PCR targeting.[3] For each target gene the *oriT-aac(3)IV* cassette was amplified from pIJ773 using the following primers (see Table S1 for primer sequences): ER2KOF and ER2KOR for *pac21*, pac21h-5 and pac21h-6 for *pac21h*, phhA-1 and phhA-2 for *phhA*. The disruptions cassette targeting *pac21* were electroporated into *E. coli* BW25113/pIJ790/cosmid 2H-5, and the disruption cassettes for *pac21h* and *phhA* were electroporated into *E. coli* BW25113/pIJ790/cosmid 5-E10. The correct insertion of the disruption cassette was verified by PCR (Figure S1) and restriction digest (data not shown). The mutagenised cosmids were introduced

into *S. coeruleorubidus* by conjugation from *E. coli* ET12567/pUZ8002. Exconjugants were selected on MS agar (20 g L^{-1} mannitol, 20 g L^{-1} soya flour, 20 g L^{-1} agar) containing 10mM $MgCl_2$ with soft TSB agar overlays containing nalidixic acid and apramycin.[2] Selection of apramycin-resistant, kanamycin-sensitive exconjugants was performed on ISP2 agar (4 g L^{-1} yeast extract, 10 g L^{-1} malt extract, 4 g L^{-1} glucose, 15 g L^{-1} agar, pH 7.2) containing the appropriate antibiotics. The double cross-over mutants were verified by PCR with gDNA as template (Figure S1).

Genetic complementation. For genetic complementation in *Streptomyces*, genes were cloned into the *Nde*I and *Hin*dIII sites of pIJ10257.[4] Genes of interest were PCR amplified using primers pac21-1 and pac21-3 for *pac21*, pac21h-3 and pac21h4 for *pac21h*, mTyr-3 and mTyr-4 for *phhA*, and pac21h-3 and mTyr-4 for *pac21h-phhA*. PCR products were directly subjected to restriction digest with the appropriate enzymes or passaged through pJET1.2 (Fermentas, York, UK) prior to ligation into linearised pIJ10257. The resulting pIJ10257 derivatives were introduced to *Streptomyces* by conjugation as described above but using hygromycin for selection.

Heterologous expression of the pacidamycin biosynthetic gene cluster. Preparation of cosmid 2H-5 for heterologous expression has been described elsewhere.[5] The disruption cassette was excised from cosmid 2H5-KO (Table S2) by FLP-mediated recombination. This leaves a 91 bp in-frame scar. The resulting cosmid was then prepared for heterologous expression as described for 2H-5 to give pSG90. Cosmids 2H-5 and pSG90 were conjugated into *S. lividans* TK24 to give RG-4289 and RG-5275, respectively (Table S3).

Culturing and metabolite extraction. Starter cultures of *S. coeruleorubidus*, inoculated from spore stocks, were grown for 2 days at 28°C with shaking in ISP2 medium (4 g L^{-1} yeast extract, 10 g L^{-1} malt extract, 4 g L^{-1} glucose, pH 7.2) containing the appropriate antibiotics as required. The main culture (ISP2) was inoculated with 5% starter culture and grown for 5 days at 28°C with shaking in the absence of antibiotics. Where appropriate, sterile solutions of amino acids were added to the main culture at the start. The feeding of L-2-chlorophenylalanine (2-Cl-Phe) was performed as previously described.[6] Starter cultures of *S. lividans* strains were grown for 2 days in 2×YT (16 g L^{-1} tryptone, 10 g L^{-1} yeast extract, 5 g L^{-1} NaCl) with the appropriate antibiotics as required. The main culture (Medium B: 20 ml L^{-1}

glycerol, 2.5 g L^{-1} glycine, 1 g L^{-1} NaCl, 1 g L^{-1} KH_2PO_4, 0.1 g L^{-1} $CaCO_3$, 0.1 g L^{-1} $FeSO_4$, 0.1 g L^{-1} $MgSO_4$ $7H_2O$, pH 7.0) was inoculated with 5% starter culture and incubation continued at 28°C with shaking in the absence of antibiotics. Metabolites were extracted from cell-free broth (10 ml) using XAD-16 resin and eluted from the resin with methanol. The dried residue was redissolved in 200 µl water:methanol 1:1 and subjected to LC-MS analysis.

Heterologous expression and purification of PhhA. The *phhA* gene was PCR-amplified using *Pfu* polymerase (from cosmid 5-E10 using primers mTyr-3 and mTyr-4, Table S1). The *Nde*I-*Hin*dIII digested PCR product was ligated into pET-28b(+) (Merck Biosciences, Nottingham, UK) to allow for expression of PhhA as a N-terminal His-tagged fusion protein. The construct was verified by DNA sequencing. *E. coli* BL21(DE3) was used as expression host in Luria Broth containing 50 µg mL^{-1} kanamycin. Protein production was induced with 80 µM IPTG and carried out at 16°C for 24 hours. Harvested cells were resuspended in lysis buffer (0.5 M NaCl, 50 mM Tris-HCl, 10 mM imidazole, pH 8.0) supplemented with 100 µM ammonium iron(II) sulphate and 2 mM DTT. After cell lysis by sonication and removal of cell debris the soluble fraction was loaded onto Ni-NTA resin (Qiagen). The resin was washed with lysis buffer and eluted with lysis buffer containing 0.4 M imidazole. Protein containing fractions were pooled. PhhA was further purified on a HiLoad 16/60 Superdex 200 column (GE Healthcare, Little Chalfont, UK) in 20 mM HEPES, 200 mM NaCl, pH 7.5. His_6-PhhA elutes as a monomer. Protein-containing fractions were treated with glycerol to give 10% final concentration and, where necessary, concentrated using Amicon Ultra centrifugal filters (MWCO 10 kDa). Protein was flash-frozen and stored at -80°C. The purity was assessed by SDS-PAGE analysis (Figure S2). Protein concentrations were determined by the Bradford method using BSA as the standard. Approximately 3 mg of His_6-PhhA was isolated from a 1 L culture.

Enzyme assays. PhhA assays contained 0.5-5 µM PhhA, 1 mM L-Phe,, 5 mM DTT and 84 U $µl^{-1}$ catalase in 50 mM HEPES, pH 7.5. Reactions were started by the addition of 0.1 mM 6,7-dimethyltetrahydropterin and incubated at 28°C. The enzyme was then removed by filtration through Amicon spin filters (MWCO 10 kDa). The filtrate was analysed by HPLC. Compounds were separated on an Agilent Eclipse XDB-C8 column (5 µm, 4.6x150 mm) with 50 mM triethylamine, 60 mM

trifluoroacetic acid as buffer A and methanol as solvent B. The gradient was: 0-3 min 3% B, 3-11 min 3-30% B, 11-12 min 30-60% B, 12-15 min 60% B, 15-18 min 60-30% B, 18-23 min 3% B. Tyrosine-optimised conditions were used for detection by fluorescence detector (ex 270 nm, em 305 nm). Time course experiments were essentially carried out as described above except that analysis was carried out in Nunc 96-well plates (100 µl reaction volume) using a Molecular Devices SpectraMax M5 instrument. The change in fluorescence emission at 305 nm was followed using an excitation wavelength of 270 nm.

LC-MS analysis. For routine analysis, compounds were separated on a Waters XBridge^{TM} C18 column (3.5 µm, 2.1x150 mm) using 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B for the following gradient: 0-0.5 min 10% B, 0.5-9 min 10-95% B, 9-11 min 95% B, 11-11.5 min 95-10% B, 11.5-14 min 10% B. The flow rate was 0.35 ml min^{-1}. Compounds were mass analysed by a Shimadzu LCMS-2010 single quad using electrospray ionisation (detector voltage 1.3 kV, CDL temperature 200°C). MS/MS analysis was performed as service (John Innes Centre) on a Thermo Finnigan LCQ DecaPlus^{XP} ion trap instrument as previously described.[6]

Table S1 Primers used in this study.

Name	Sequence 5' → 3'
5e10-4	gtcactgcgcgacttcgctg
5e10-5	caaggacatggtgctcggcaac
ER2KOF	gaagtttcacgcactacttcgagctgcaggacagacggtattccggggatccgtcgacc
ER2KOR	cgtcgaggtcgacgaaccgaccttcttcgtcagccggattgtaggctggagctgcttc
mTyr-3	ccatatgcaagggccgcacgccca
mTyr-4	caagcttggctcagtgaggtgtcaccgagc
pac21-1	ggaattccatatgtcagtacagttcggtgcgccg
pac21-3	cccaagcttgatgccgacgccttgctcaacc
pac21h-3	gggaattccatatgtctctcacattggtcgagcg
pac21h-4	ctagaagcttcagccaggcaactcctccg
pac21h-5	gagagataggggttggcaaggtttatgtctctcacattgattccggggatccgtcgacc
pac21h-6	gggctctcccggtcggcgcctcgctcagccaggcaactctgtaggctggagctgcttc
pac21KOconfirm-fw	atacttcctttcgtgtcctgg
pac21KOconfirm-rev	tagaacacgaccagctccgtt
phhA-1	cgtcttgaattggagtgatccctcatgcaagggccgcacattccggggatccgtcgacc
phhA-2	acgcgcctcccggaagaacccggctcagtgaggtgtcactgtaggctggagctgcttc

Table S2 Plasmids and cosmids used in this study.

Name	Parent	Description / Insert	Reference
2H-5	Supercos	cosmid containing pacidamycin gene cluster	[5]
5-E10	Supercos	Cosmid containing *pac21-phhA*	this study
pIJ790	pKD20	λ RED recombination plasmid	[3]
pIJ773		template for *oriT-aac(3)IV* replacement cassette	[3]
BT340		FLP recombination plasmid	[3]
pIJ10257		*Streptomyces-E. coli* shuttle plasmid	[4]
2H5-KO	2H-5	*pac21::oriT-aac(3)IV*	this study
pSG89	2H5-KO	Δ*pac21*	this study
pSG90	pSG89	*aph::oriT-aac(3)IV-attP-int ΦC31*	this study
pSG124	5-E10	*pac21h::oriT-aac(3)IV*	this study
pSG125	5-E10	*phhA::oriT-aac(3)IV*	this study
pSG107	pIJ10257	*pac21*	this study
pSG109	pIJ10257	*pac21h*	this study
pSG121	pIJ10257	*phhA*	this study
pSG123	pIJ10257	*pac21h-phhA*	this study
pSG120	pET-28a(+)	*phhA*	this study

Table S3 Strains used in this study.

Name	Description	Reference
E. coli BW25113	Host for λ RED-mediated recombination	[3]
Streptomyces coeruleorubidus AB1183F-64	Wild type, pacidamycin producer	[5]
Streptomyces lividans TK24	*str-6* SLP2^{-} SLP3^{-}	
RG-4289	*S. lividans* + 2H-5	[5]
RG-5275	*S. lividans* + pSG90	this study
RG-5359	RG-4289 + pSG109	this study
RG-5360	RG-5275 + pSG109	this study
RG-5364	RG-4289 + pSG121	this study
RG-5366	RG-5275 + pSG121	this study
RG-5365	RG-4289 + pSG123	this study
RG-5367	RG-5275 + pSG123	this study
RG-4028	*S. coeruleorubidus pac21::oriT-aac(3)IV*	this study
RG-5381	*S. coeruleorubidus pac21h::oriT-aac(3)IV*	this study
RG-5398	RG-5381 + pSG109	this study
RG-5382	*S. coeruleorubidus phhA::oriT-aac(3)IV*	this study

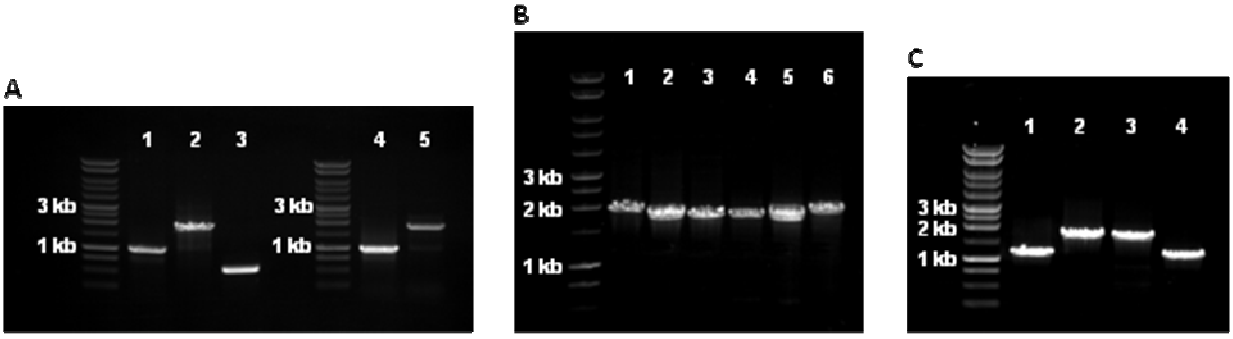


Figure S1 Verification of gene knock-outs. Relevant bands of the DNA size marker have been labeled.

A PCR analysis of *pac21* knock-outs using primers pac21KOconfirm-fw and -rev. 1: cosmid 2H-5, 2: cosmid 2H5-KO, 3: cosmid pSG89/pSG90, 4: *S. coeruleorubidus* wild type gDNA, 5: *S. coeruleorubidus* RG-4028 gDNA.

B PCR analysis of *pac21h* knock-outs using primers 5e10-4 and 5e10-5. 1: cosmid 5-E10, 2: mixed template 5-E10 + pSG124, 3: pSG124, 4: RG-5381 gDNA, 5: mixed template RG-5381 gDNA + *S. coeruleorubidus* wild type gDNA, 6: *S. coeruleorubidus* wild type gDNA.

C PCR analysis of *phhA* knock-outs using primers mTyr-3 and mTyr-4. 1: cosmid 5-E10, 2: pSG125, 3: RG-5382 gDNA, 4: *S. coeruleorubidus* wild type gDNA.

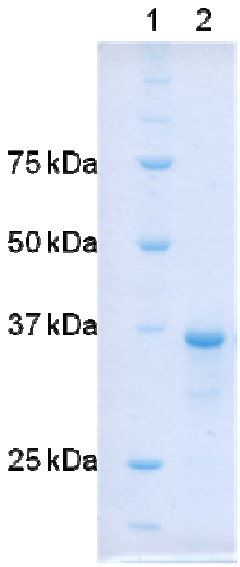


Figure S2 SDS-PAGE of purified His_6-PhhA (lane 2) with protein size marker (lane 1).

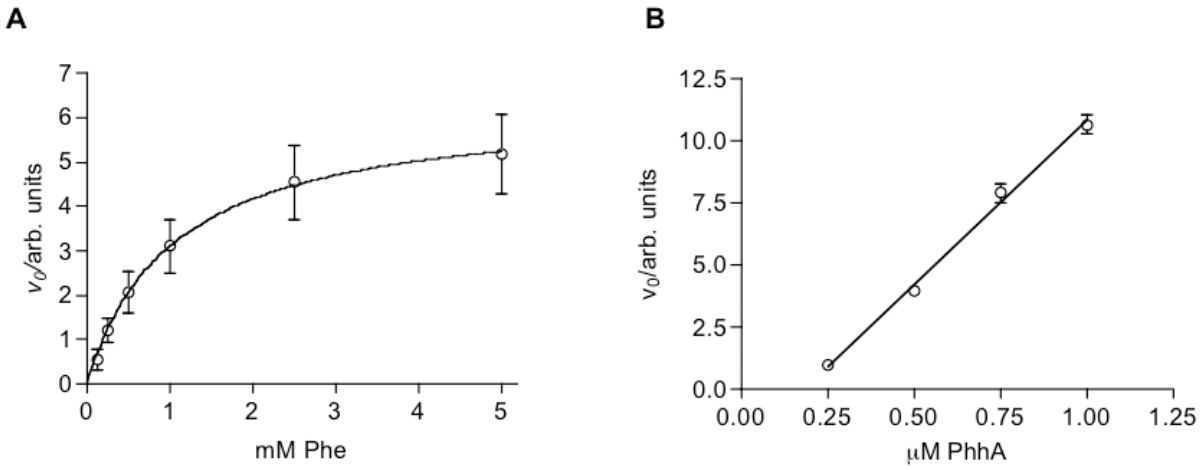
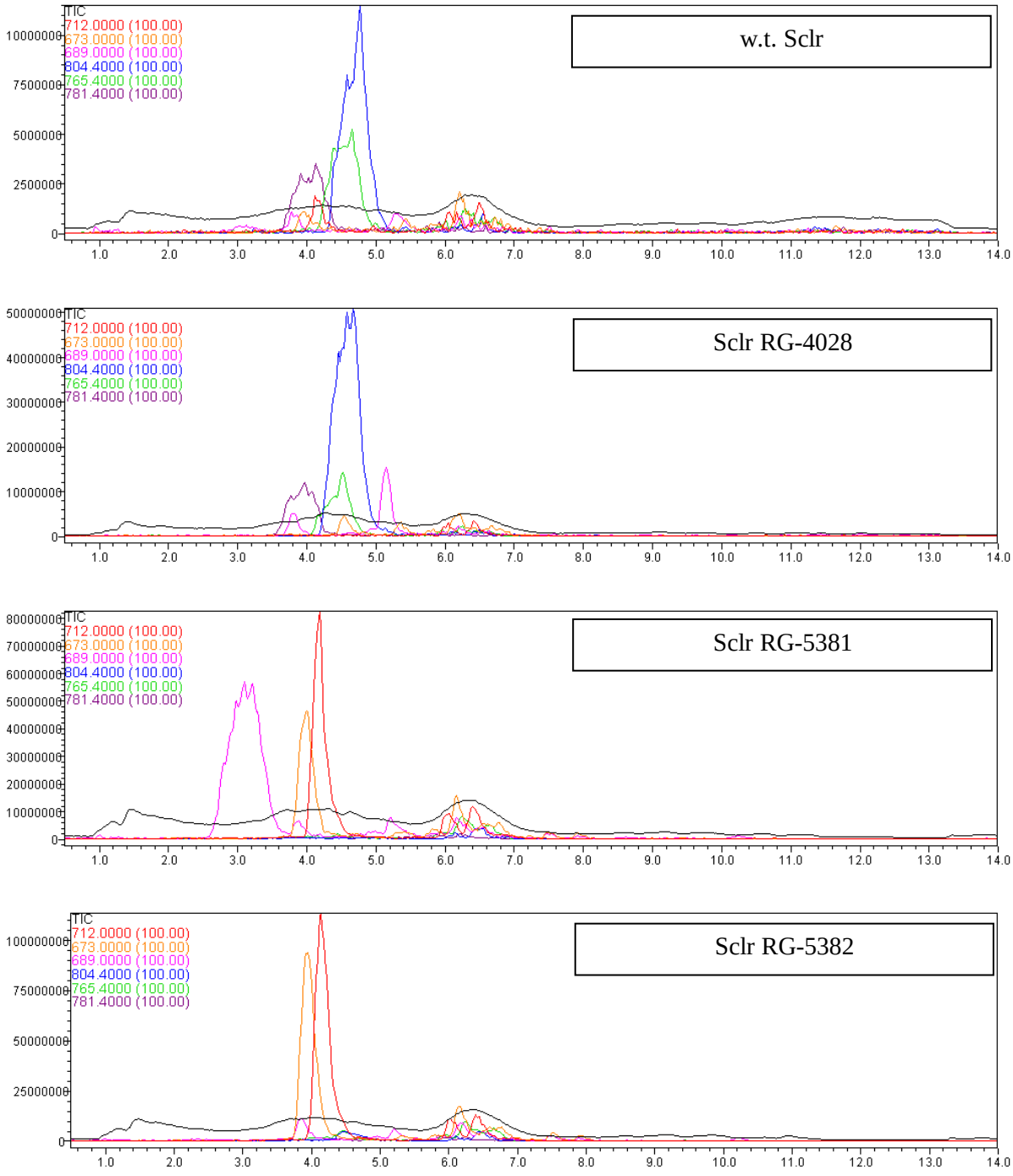
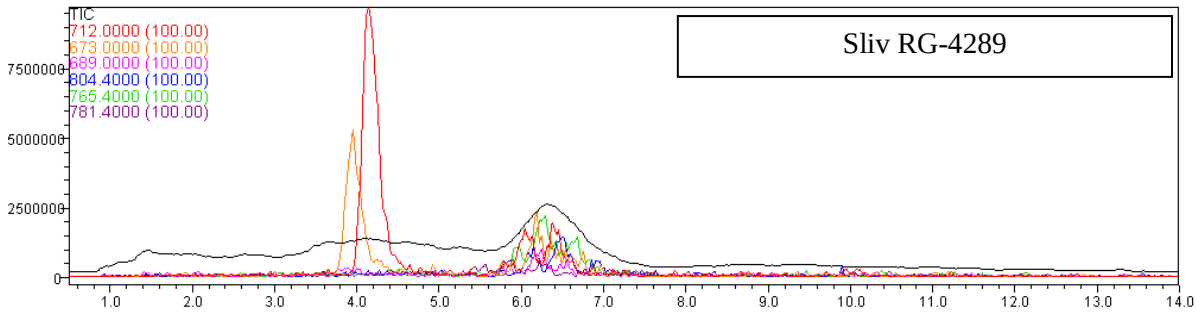


Figure S3 Kinetic analysis of PhhA reaction. A: Initial velocities (v_0) of 0.5 µM PhhA, 0.1 mM $DMPH_4$, 0.125 - 5.0 mM L-Phe, 84 U μl^{-1} catalase plotted against Phe concentration and non-linear regression ($v_0 = v_{max} \cdot [Phe] \cdot (K_m + [Phe])^{-1}$). Data derived from three independent experiments each run in triplicate. B 0.25-1.0 µM PhhA, 0.1 mM $DMPH_4$, 5 mM L-Phe, 84 U μl^{-1} catalase plotted against PhhA concentration and linear regression.

LC-MS Analysis – Extracted Ion Chromatograms

Pacidamycin D	*m/z* 712
Pacidamycin S	*m/z* 673
Pacidamycin T	*m/z* 689
Pacidamycin 4	*m/z* 804
Pacidamycin 5	*m/z* 765
Pacidamycin 5T	*m/z* 781

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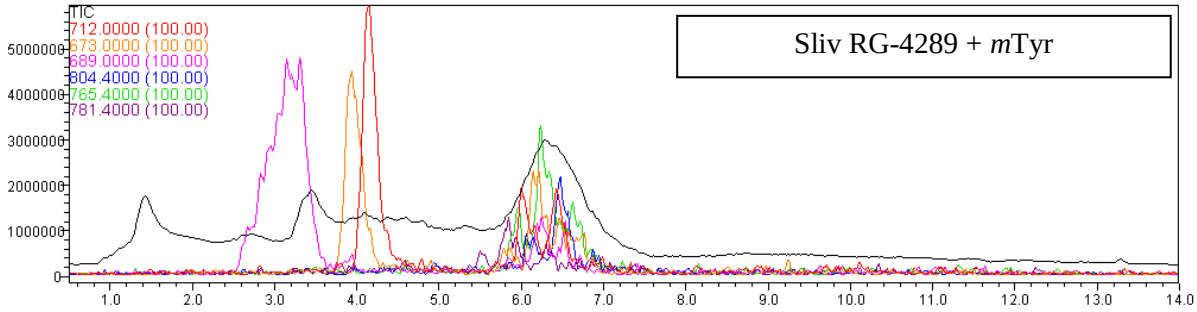
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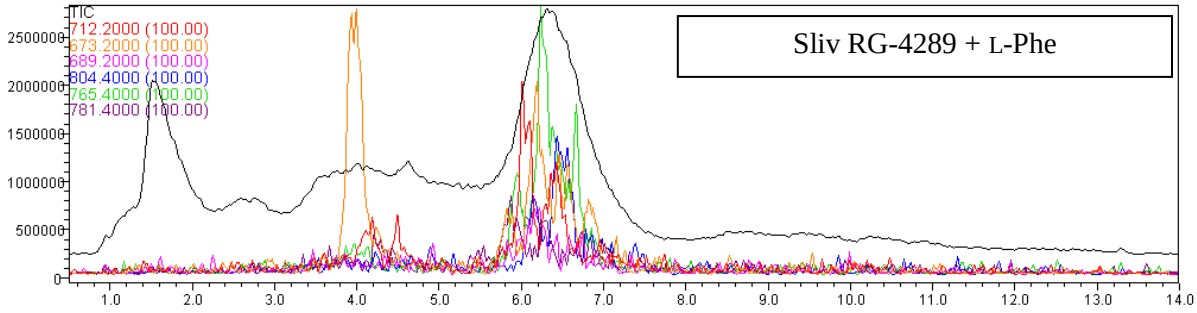
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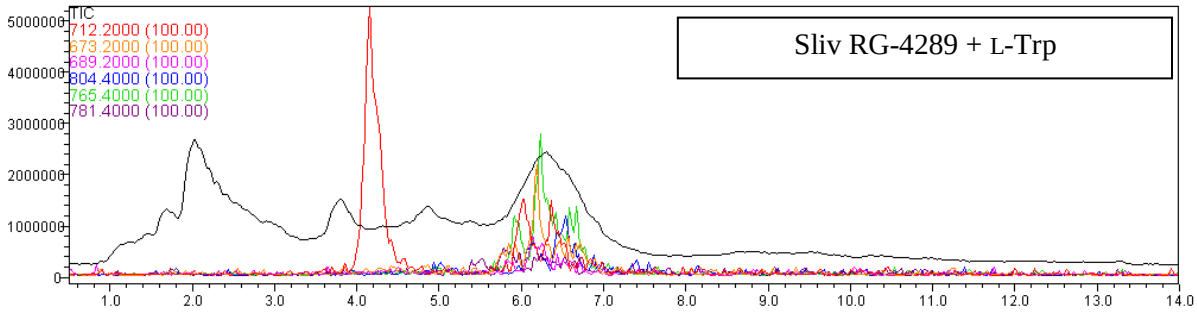
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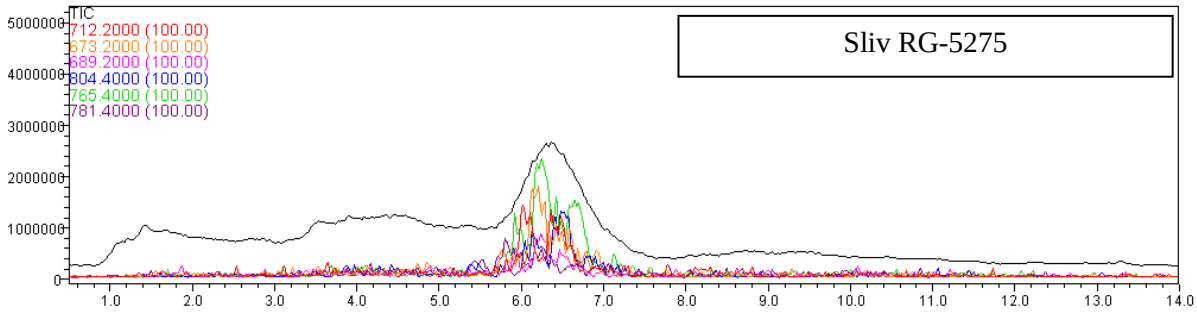
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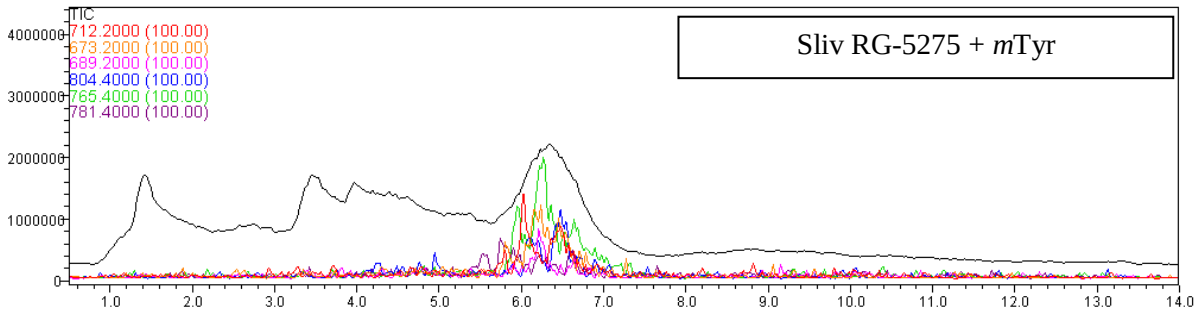
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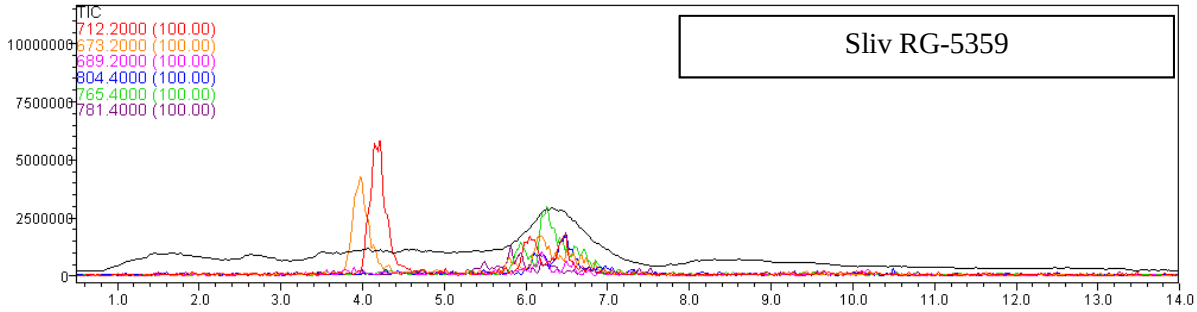
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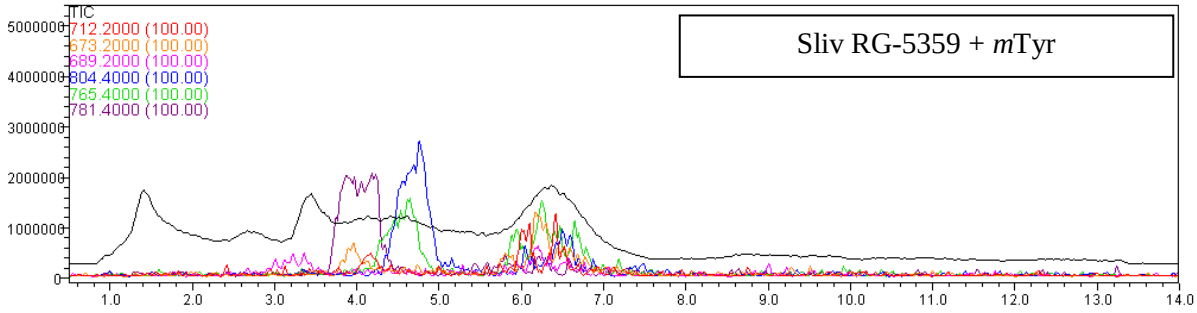
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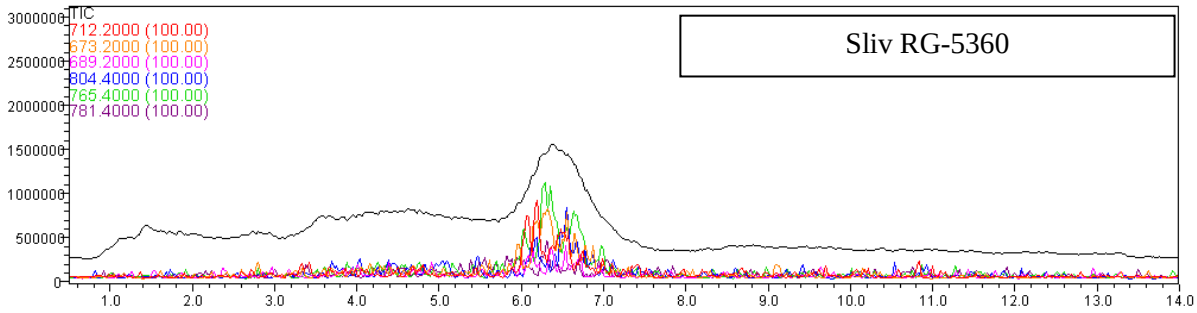
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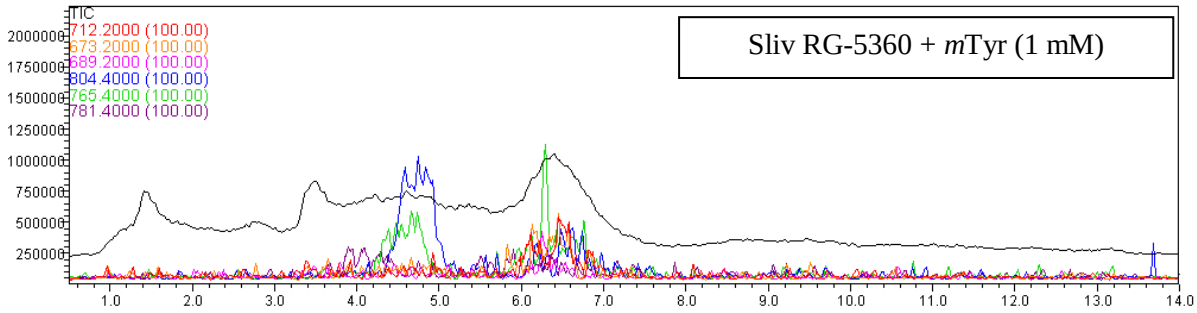
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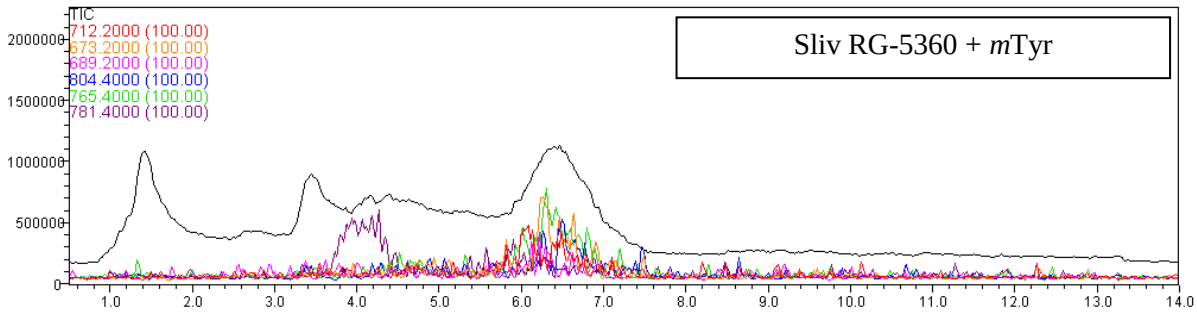
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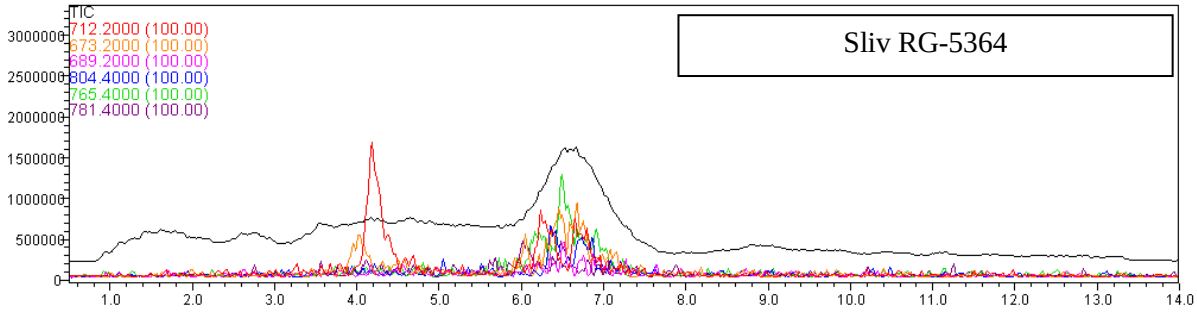
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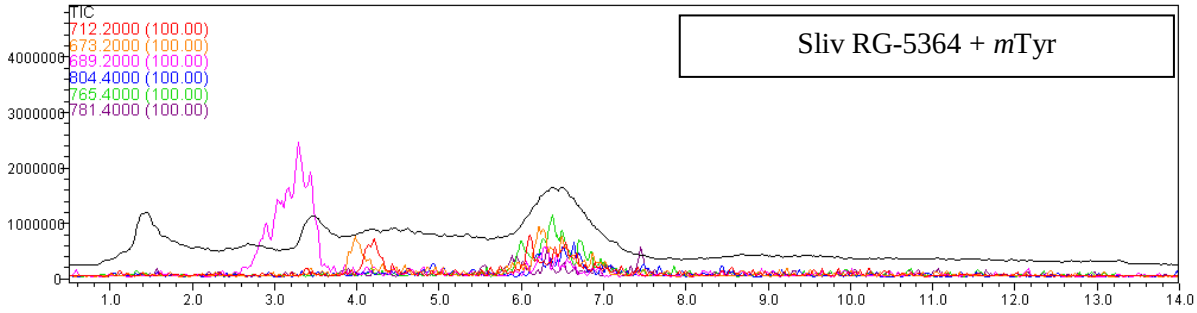
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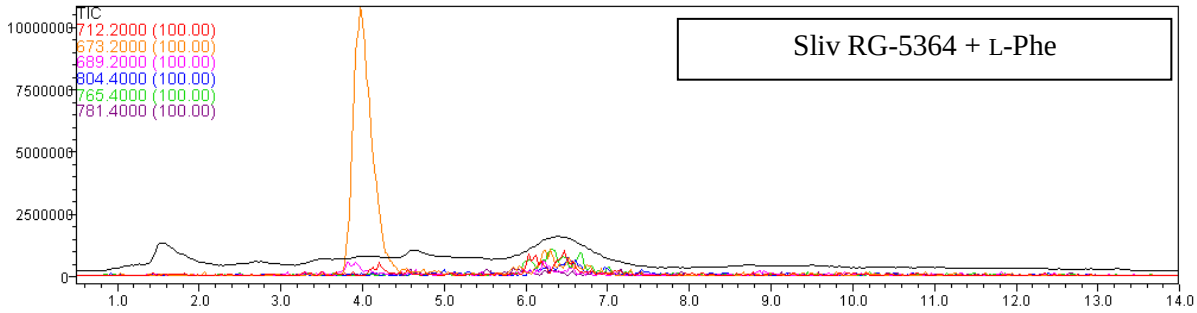
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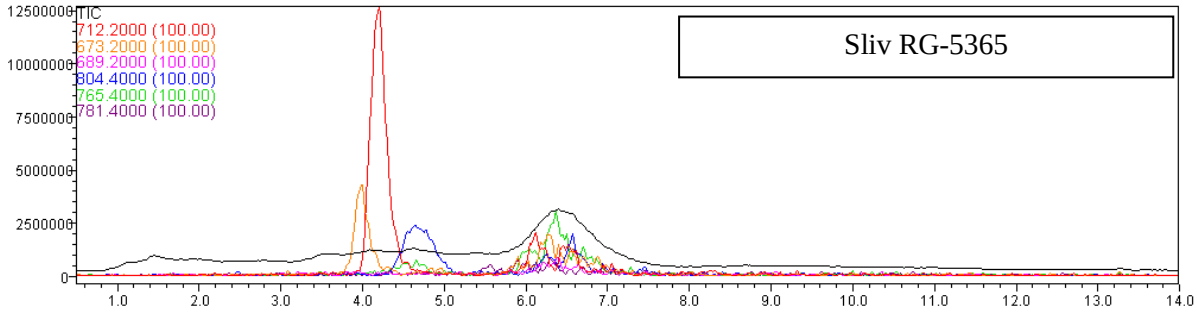
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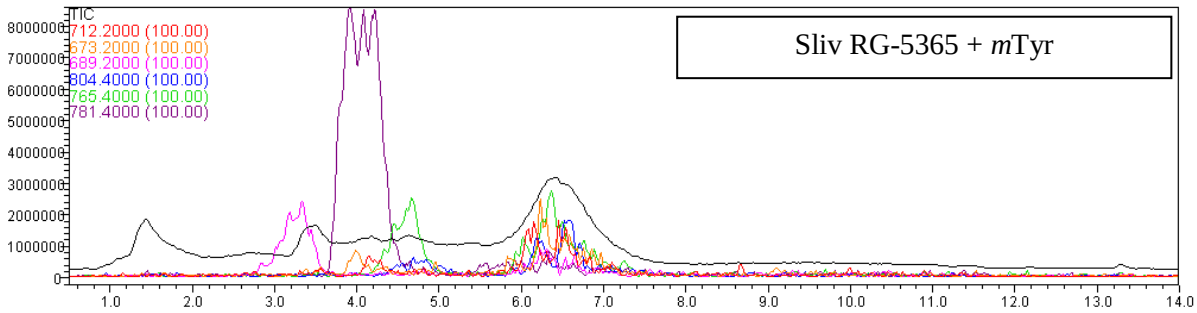
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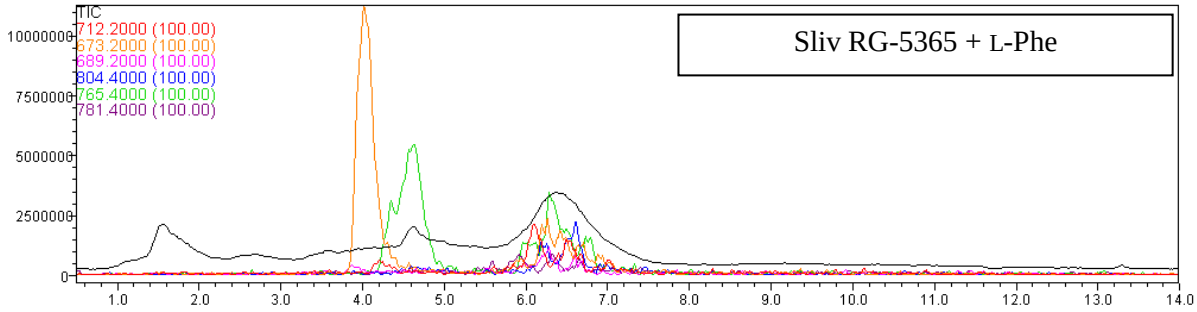
Sliv RG-5365 + mTyr


TIC
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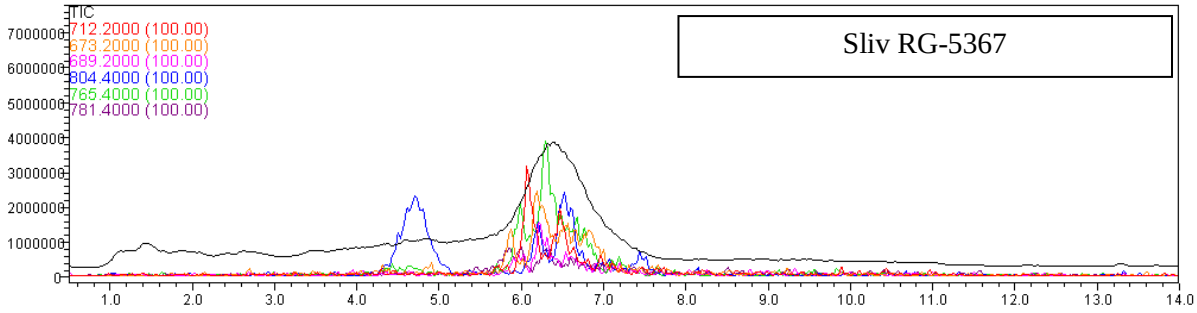
673.2000 (100.00)
689.2000 (100.00)
804.4000 (100.00)
765.4000 (100.00)
781.4000 (100.00)
Sliv RG-5365 + L-Phe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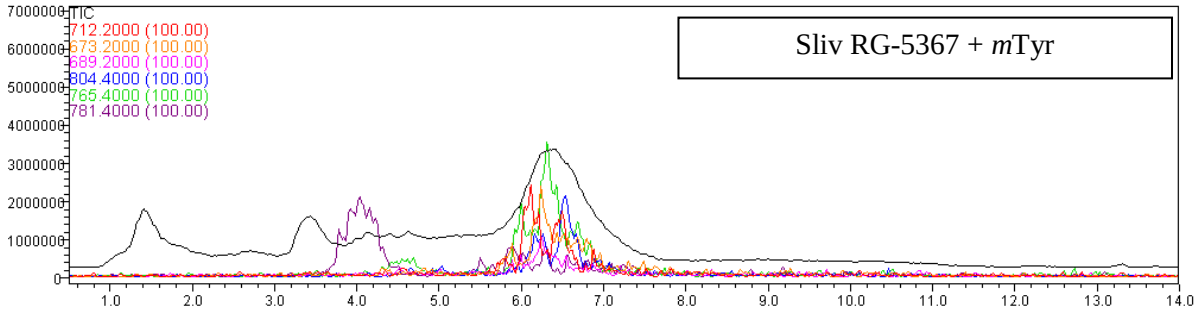
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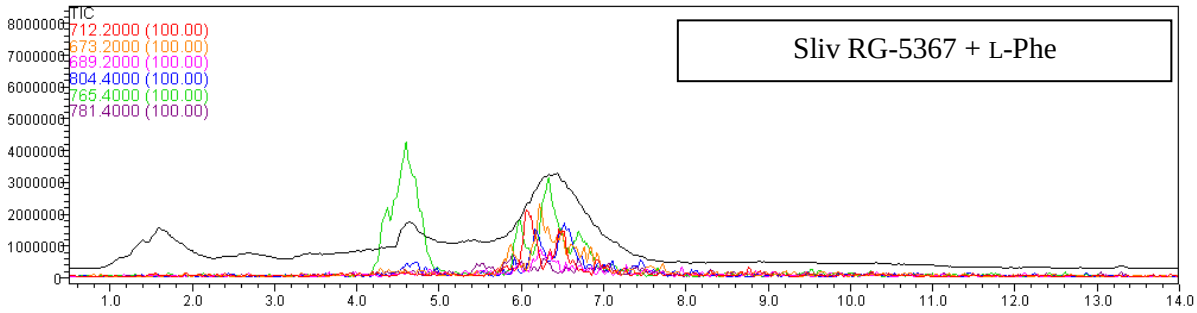
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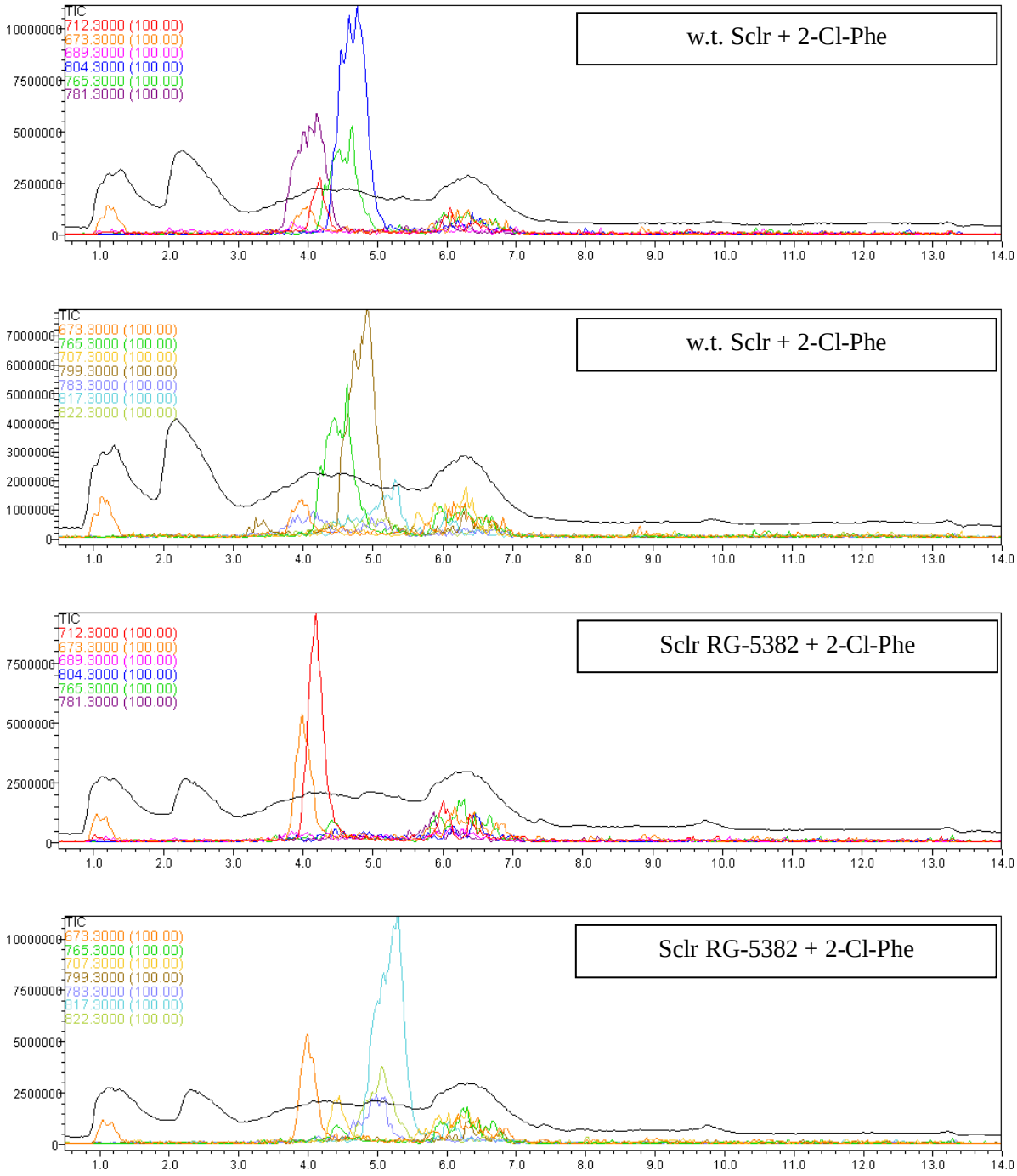
Sliv RG-5367 + L-Phe

2-Chlorophenylalanine Feeding



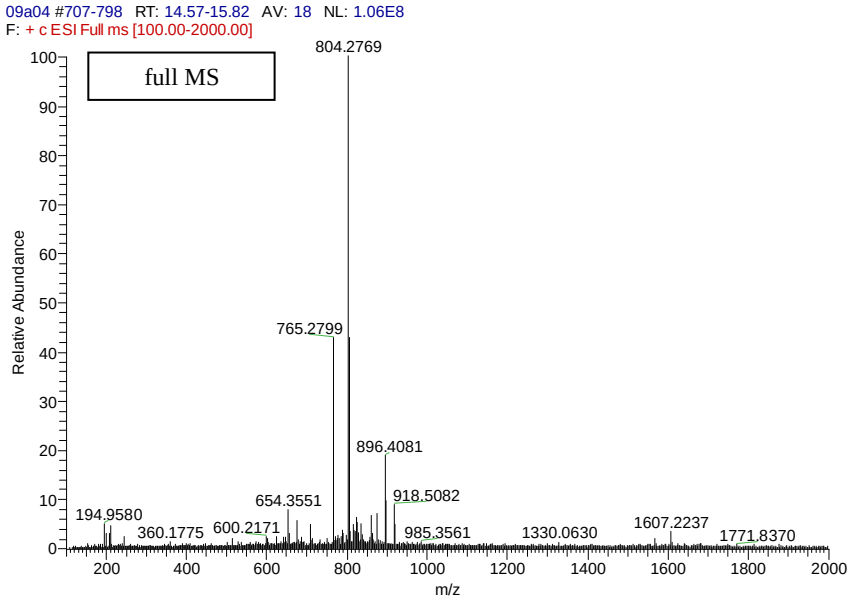
Pacidamycin D	*m/z* 712	Pacidamycin 4	*m/z* 804
Pacidamycin S	*m/z* 673	Pacidamycin 5	*m/z* 765
Pacidamycin T	*m/z* 689	Pacidamycin 5T	*m/z* 781

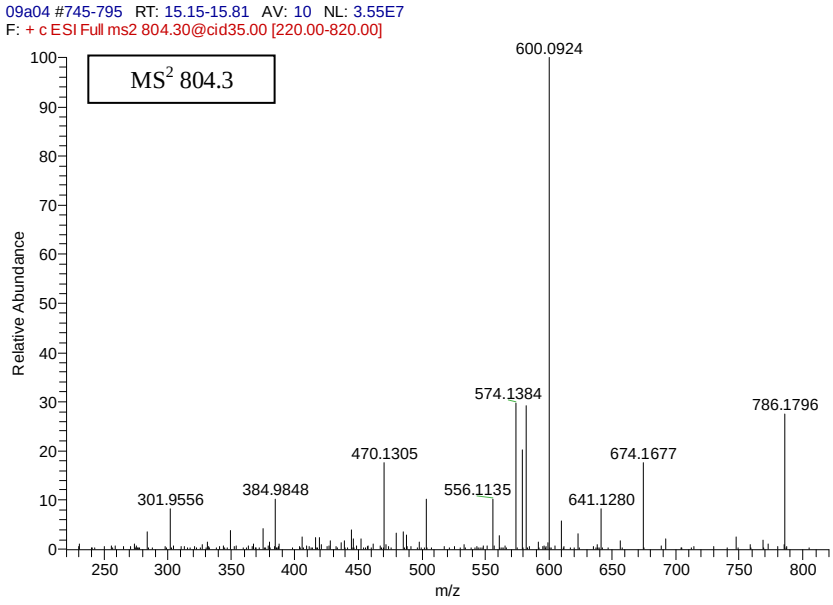
C-Cl-Pacidamycin	*m/z* 707
OH-Cl-Pacidamycin	*m/z* 799
N-Cl-Pacidamycin	*m/z* 783
Cl_2-Pacidamycin	*m/z* 817
N-Cl-Pacidamycin 4	*m/z* 822

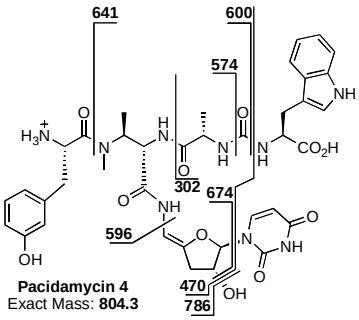
R_1 = *m*Tyr, R_2 = 2-Cl-Phe or R_1 = 2-Cl-Phe, R_2 = *m*Tyr

MS/MS Spectra of Chloropacidamycins

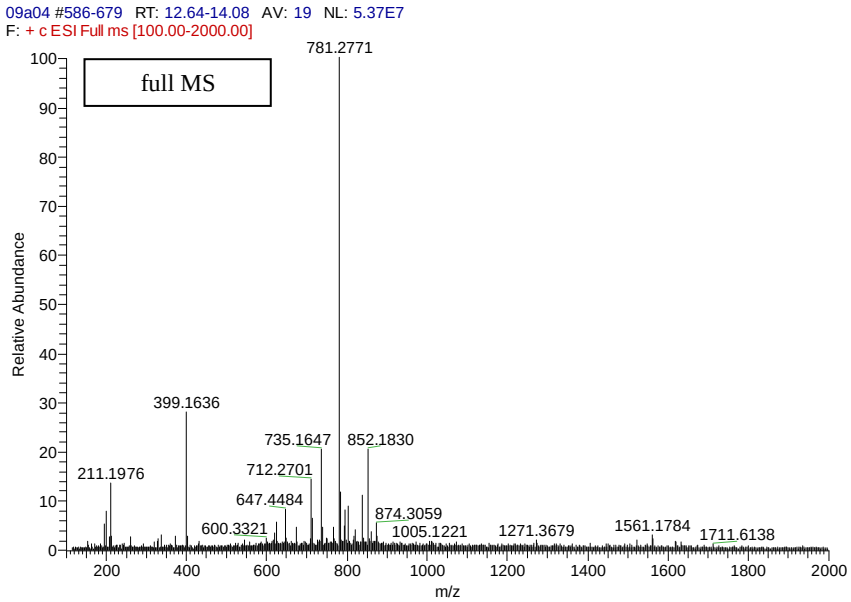
w.t. Sclr + 2-Cl-Phe

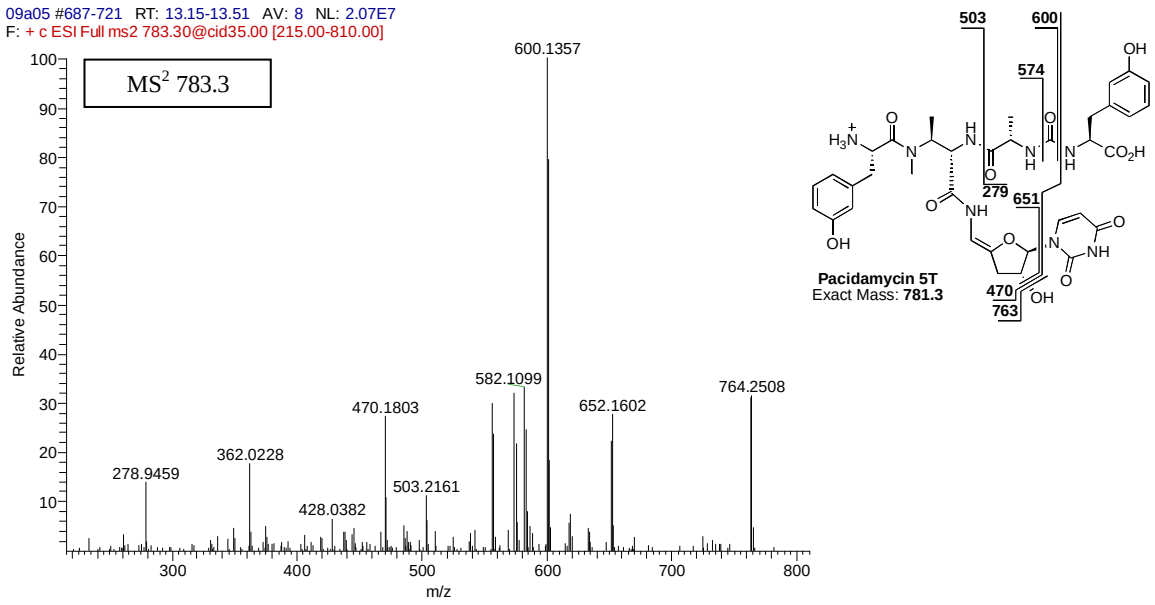






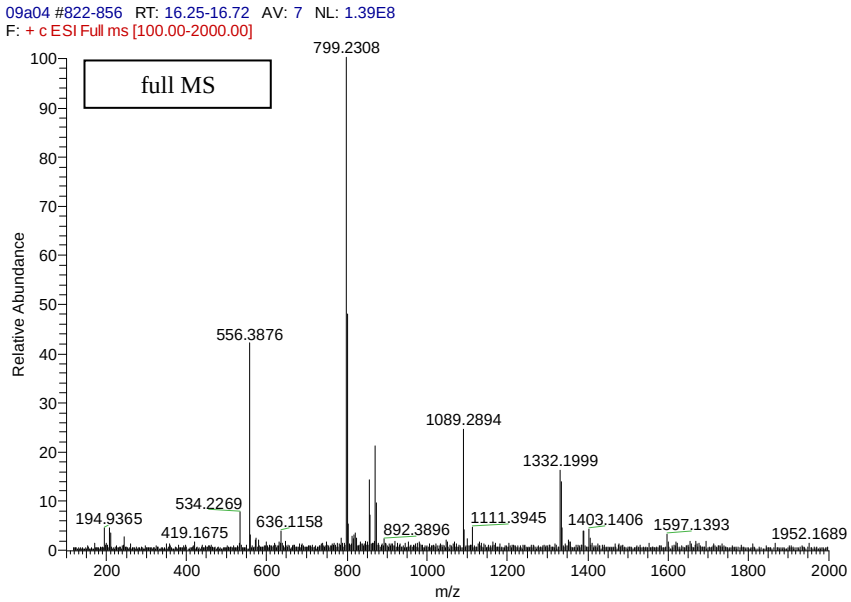
w.t. Sclr + 2-Cl-Phe

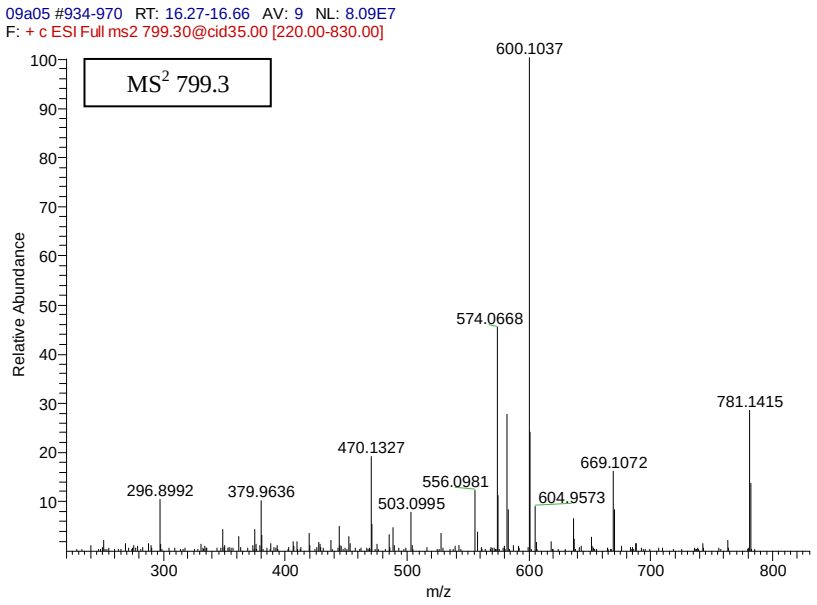


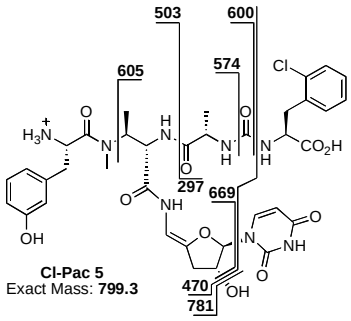


isotope of pacidamycin 5T (RT ~13.3)
NOT *m/z* 783 N-Cl-Pac (RT ~17.5 min)

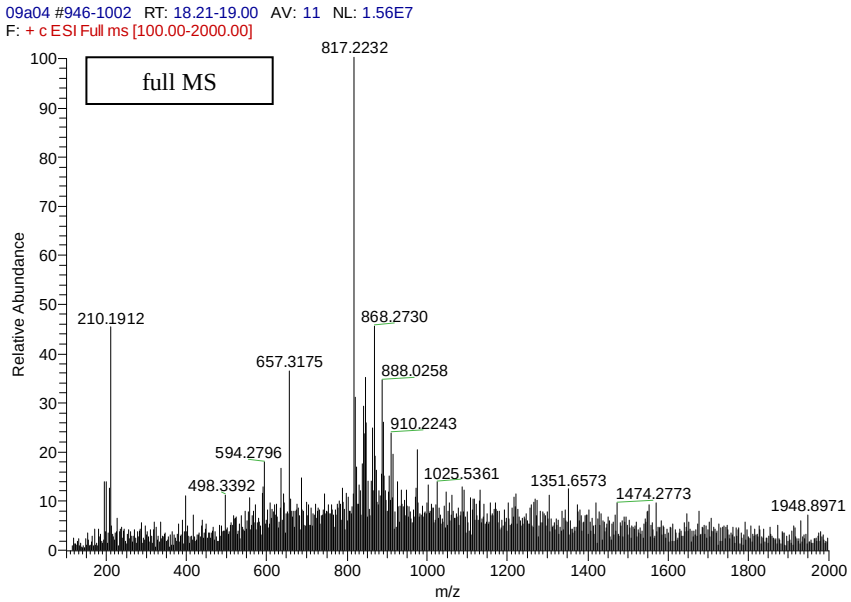
w.t. Sclr + 2-Cl-Phe

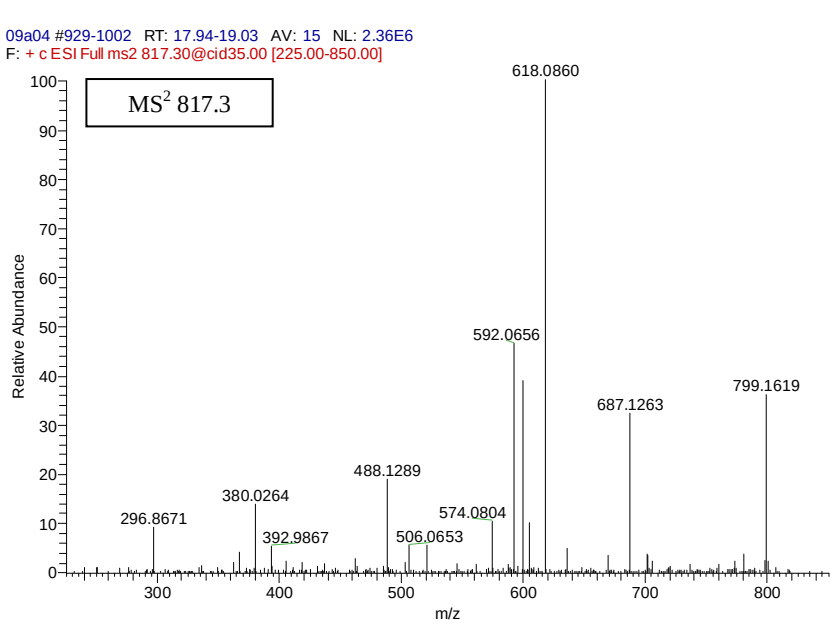


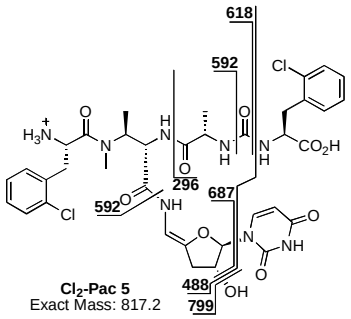




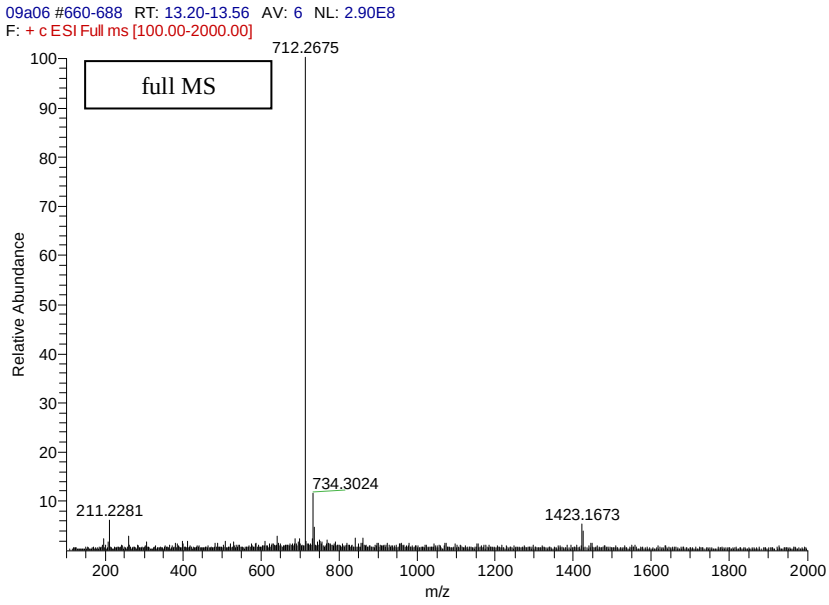
w.t. Sclr + 2-Cl-Phe

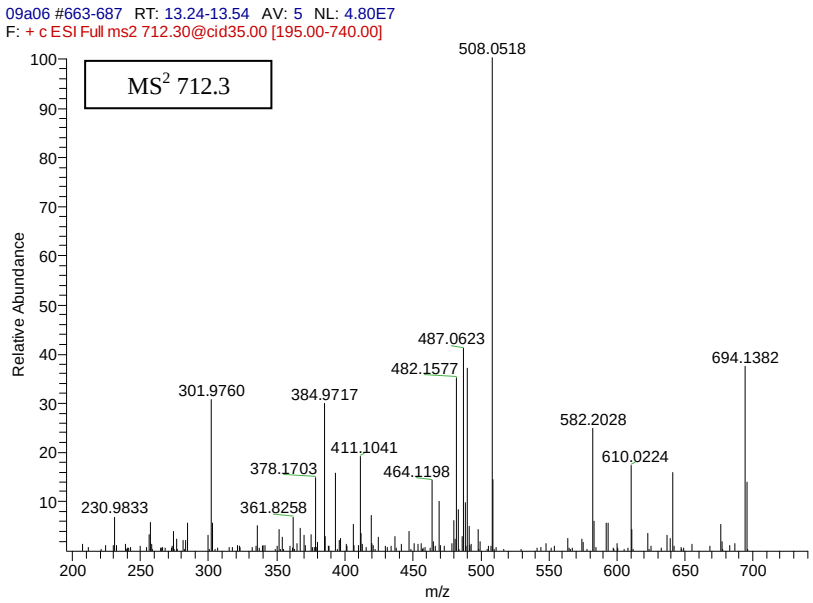


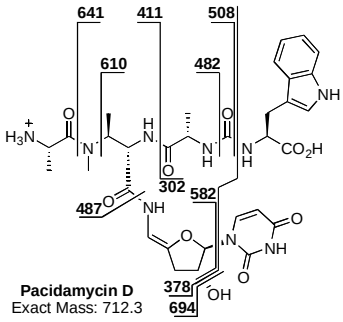




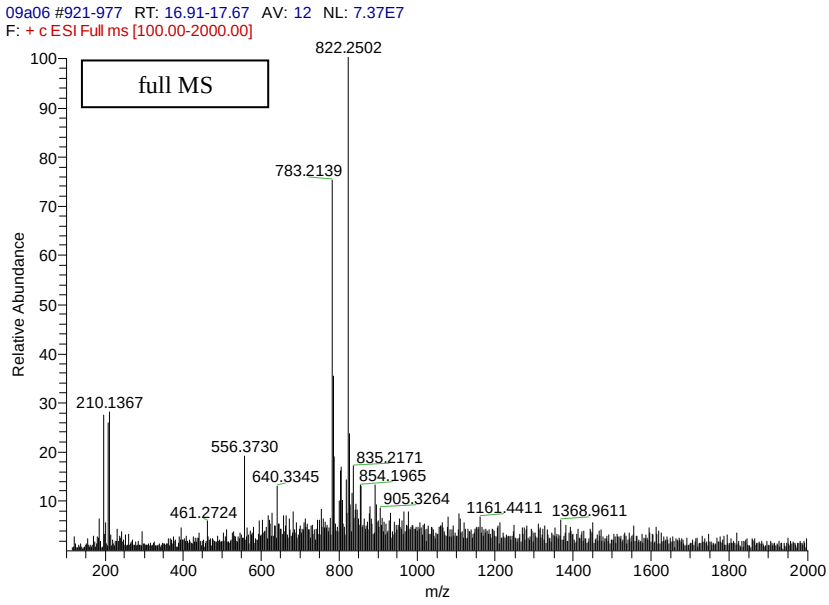
Sclr RG-5382 + 2-Cl-Phe

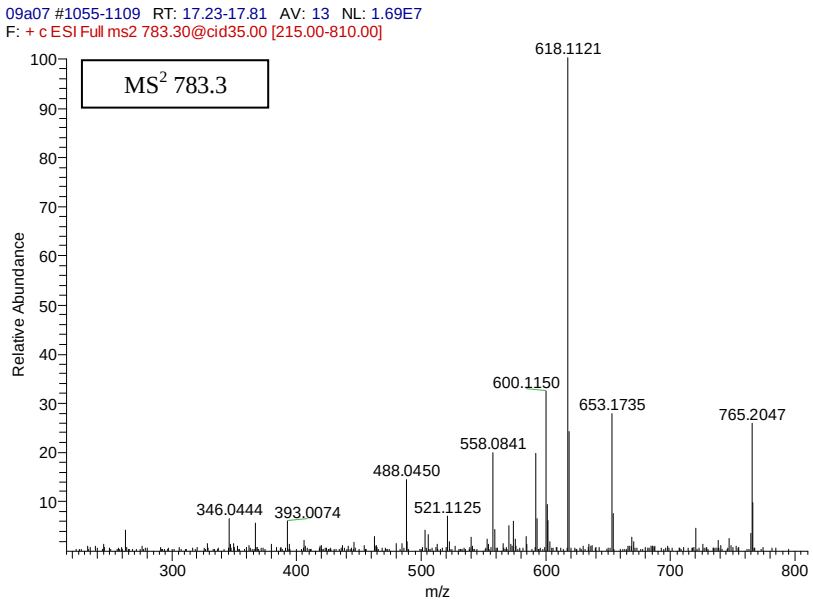


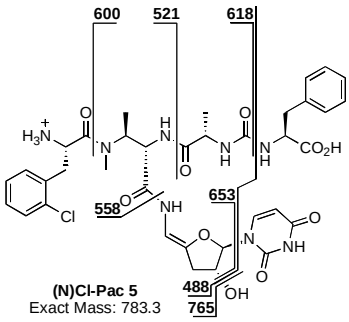




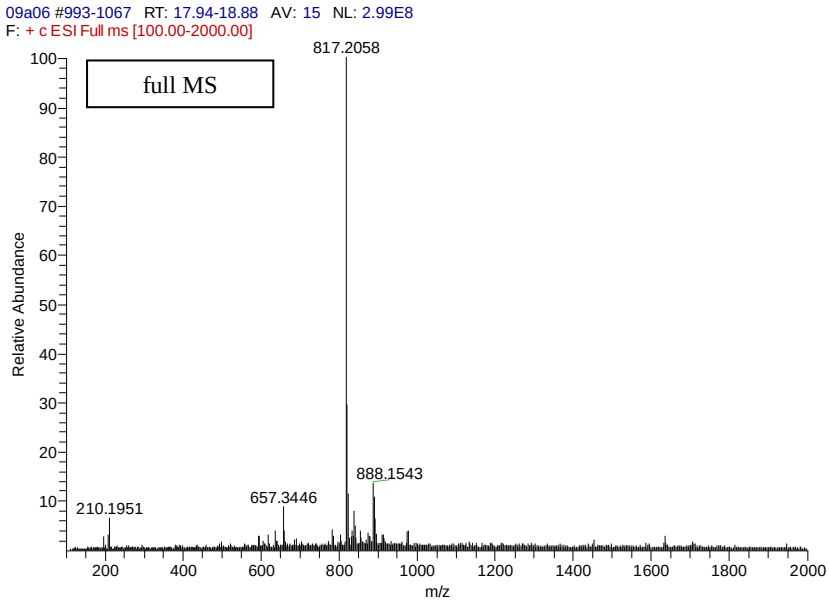
Sclr RG-5382 + 2-Cl-Phe

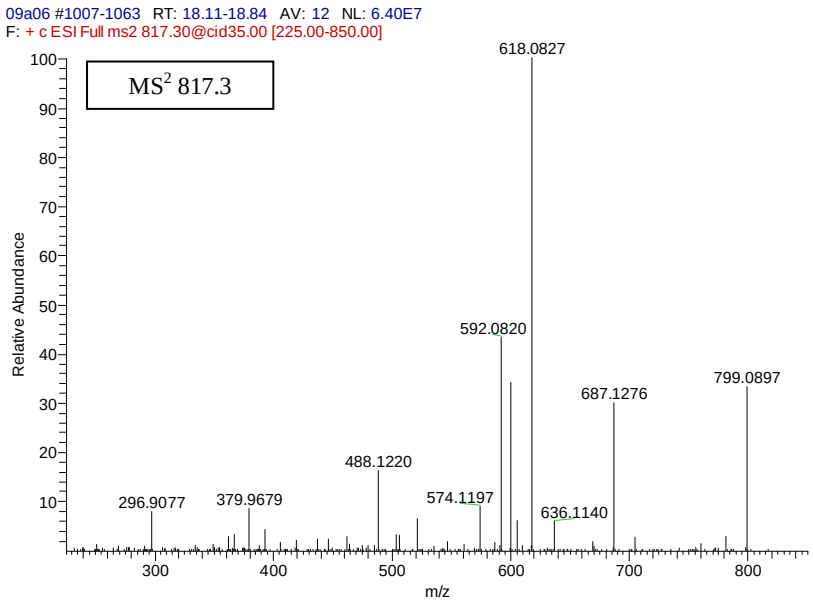


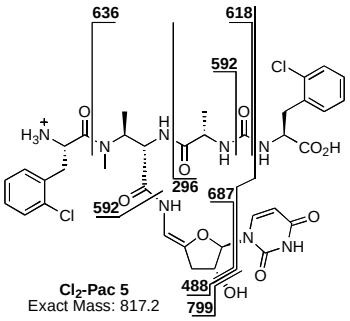




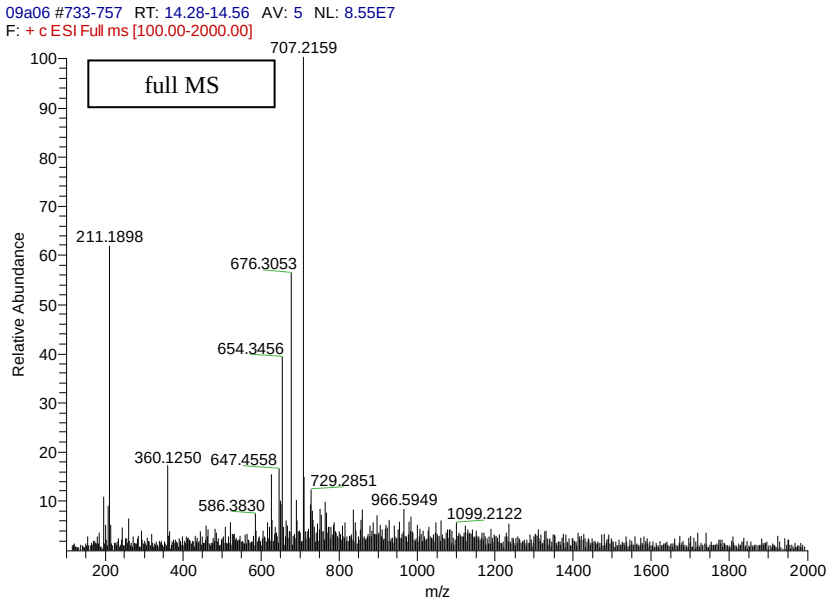
Sclr RG-5382 + 2-Cl-Phe

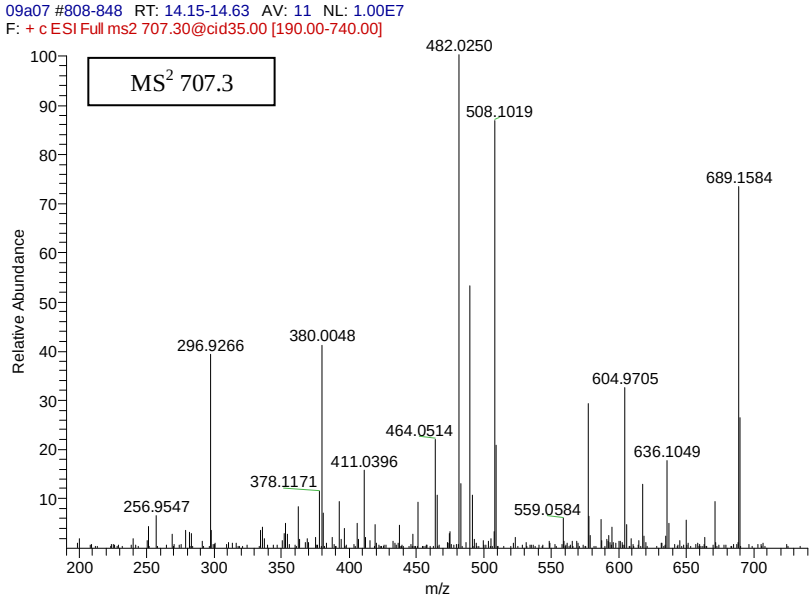


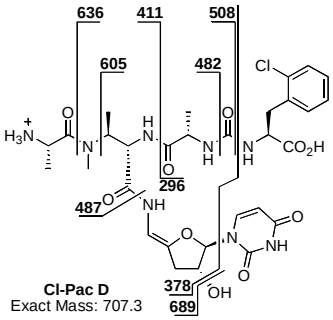




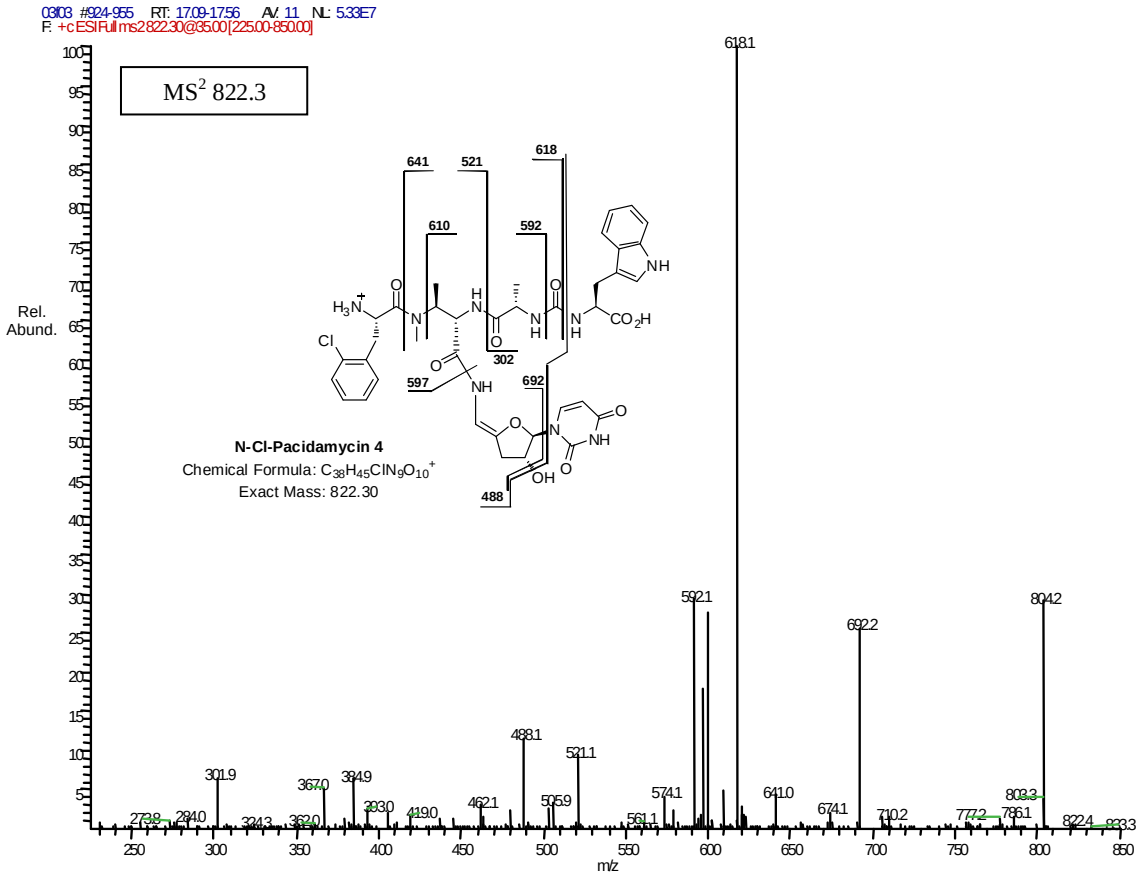
Sclr RG-5382 + 2-Cl-Phe







Sclr RG-5382 + 2-Cl-Phe



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