Electronic Supplementary Information

In trans hydrolysis of carrier protein-bound acyl intermediates by CitA during citrinin biosynthesis

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Experimental Details

Materials and Cloning

All reagents were purchased from Sigma-Aldrich unless described otherwise. Buffer stocks (10X) were made from mixing KH_2PO_4 and K_2HPO_4 salts, and pH was adjusted with KOH at 25 °C. Commercially obtained malonyl-CoA and acetoacetyl-CoA were re-purified to minimize free CoA and acetyl-CoA. Purification was performed on an Agilent 1100 HPLC using a Phenomenex Kinetex C_{18} column (5 μm, 250 x 10 mm) with 100% H_2O + 0.1% TFA (v/v) for 2 min followed by 0-35% MeCN/H_2O + 0.1% TFA over 23 min at 4 mL/min. Purified CoAs were lyophilized and stored at -80 °C. [1-^{14}C]-acetyl-CoA was purchased from Perkin Elmer and used without further purification. *M. purpureus* NRRL 1596 was obtained from the Agricultural Research Service, USDA. Mycelia were cultured on PDA plates and gDNA was purified using the DNeasy Plant mini Kit (Qiagen). The coding sequence for CitA was amplified from purified gDNA using primers MpOrf4-g5 and MpOrf4-g3 and Phusion polymerase (New England Biolabs) and ligated into pCR-Blunt vector using the Zero Blunt PCR Cloning Kit (Invitrogen). An expression insert reflecting the re-annotated start codon was amplified from this plasmid using primers MpOrf4-5.2 and MpOrf4-3 and Phusion polymerase. The insert and pET-24a (Novagen) were digested with NdeI and NotI-HF (New England Biolabs) and ligated with T4 ligase (New England Biolabs). CitA mutant expression plasmids were prepared analogous to the wild-type plasmid by overlap extension PCR using the corresponding primers in Table S1. Expression plasmids sequences were verified at the Johns Hopkins University Synthesis and Sequencing Facility.

In vitro reconstitution of CitA with PksCT

A detailed description of PksCT reconstitution reactions and reaction products was previously published.[1] Briefly, PksCT mono- and multidomain expression plasmids and CitA or CitA mutant expression plasmids were transformed into *Escherichia coli* BL21(DE3). Expression cultures were grown at 37 °C in LB medium to OD_{600} of ~0.6, cooled in an ice bath, and induced with IPTG (1 mM, GoldBio) at 18 °C. Following overnight expression, cells were harvested by centrifugation at 4000 x g and flash frozen in liquid nitrogen for storage at -80 °C or resuspended in 5 mL/g lysis buffer [50 mM potassium phosphate pH 7.5, 300 mM NaCl, 10% glycerol (v/v)] for immediate purification. The resuspended slurries were sonicated for 10 x 10 s, 40% amplitude (Vibra-Cell Ultrasonic Processor), on ice and cleared by centrifugation at 25,000 x g, 4 °C for 25 min. Lysates were batch bound to Co^{2+}-TALON for 1 h, 4 °C and purified by gravity column, washing with 10 column volumes of lysis buffer and 5 column volumes of lysis buffer + 2 mM imidazole, and eluted with 3 column volumes of lysis buffer + 100 mM imidazole. Purified proteins were dialyzed against reaction buffer [100 mM potassium phosphate pH 7.0, 5% glycerol (v/v)] flash frozen in liquid nitrogen, and stored at -80 °C until use. Protein concentrations were determined using the Bradford Assay (Bio-Rad) in duplicate with bovine serum albumin (Sigma-Aldrich) as a standard. ACP_{CT} was activated by Sfp with CoASH and $MgCl_2$ as described previously.[1] Reconstitution reactions included the listed PksCT domains at 10 μM each, with increasing concentration of CitA or CitA-S122A, along with 0.5 mM AcSNAC, 2 mM MalSNAC, 2 mM SAM, 1 mM NADPH, and 1 mM TCEP in reaction buffer to 250 μL. Reactions were run for 4 h at 25 °C before being acidified with 5 μL concentrated HCl, extracted into ethyl acetate 3 x 250 μL, and dried. The residue was dissolved in 250 μL 20% ACN/H_2O (v/v) and analyzed on an Agilent 1200 HPLC using a Phenomenex Prodigy ODS3 column (5 μm, 4.5 x 250 mm) with 5-85% MeCN/H_2O + 0.1% formic acid (v/v) for 40 min at 1 mL/min. Products were detected by diode array at 280 nm.

Radiolabel and LCMS assays of CitA activity

For radiochemical assays, purified *apo*-ACP_{CT} (50 μM) was activated with [1-^{14}C]-acetyl-CoA (100 μM) by Sfp (1 μM) in dialysis buffer supplemented with $MgCl_2$ (10 mM) for 1.5 h at room temperature. As shown in Fig. 3A, radiolabeled acetyl-*holo*-ACP_{CT} (10 μM) was incubated with CitA or CitA-S122A (10, 1, 0.1, or 0.01 μM) in reaction buffer (50 μL total) for 5 min at 25 °C. As shown in Fig. 4B, radiolabeled acetyl-*holo*-ACP_{CT} (10 μM) was incubated with CitA, CitA-R36A, or CitA-R236A (1 μM), with aliquots taken at the given time-points. Reactions were quenched with 5X SDS loading buffer (lacking β-mercaptoethanol) and separated by SDS-PAGE (16%). Gels were dried, exposed to a phosphorimager screen overnight, and scanned on a Typhoon 9410 Variable Mode Imager (Amersham Biosciences). Data were processed and analyzed using ImageJ.[2] For LCMS assays, *apo*-ACP_{CT} (100 μM) was activated with malonyl-CoA or acetoacetyl-CoA (200 μM) by Sfp (1 μM) in reaction buffer supplemented with $MgCl_2$ (10 mM) for 2 h at room temperature. Acyl-*holo*-ACP_{CT} (50 μM) was incubated with CitA (1 μM) for 30 min at 25 C, and 2.5 μL were diluted to 200 μL with 20 % MeCN/H_2O (v/v). The reactions were analyzed on a Waters ACQUITY Xevo G-2 UPLC-ESI-MS in positive ion mode by injecting 5 μL onto a BEH C_4 column with 100% H_2O + 0.1% formic acid (v/v) for 1 min followed by 0-80% MeCN/H_2O + 0.1% formic acid over 6.5 min. ACP_{CT} eluted at 6 min and detected ions at 600-2200 m/z were analyzed in MassLynx using MaxEnt deconvolution to give intact protein masses.

CitA homology model

The amino acid sequence for CitA was submitted to the CPHmodel server v3.2 and a model was built from PDB: 1YCD with a Z-score of 30.4. The homology model was submitted to the RAMPAGE server and contained five residues in outlier regions, three on the boundary of allowed conformations (Ala108, Ala158, Asp234) and two residues on loops distant from the active site (Asp167, Ala222).

Table S1: Primers used in this work

Primer Name	Sequence
MpOrf4-g5	ATGAAAGGGCAGACAGGGCTTCGC
MpOrf4-g3	CTAGGGAGCACCCGTCTGCGTTGC
MpOrf4-5.2	GCATCATATGGTCCAGACGAATTTAGAGGTGGTCG
MpOrf4-3	GCATGCGGCCGCGGGAGCACCCGTCTGCGTTGC
MpOrf4v2-S122A-5	GCTTGGATTTGCACAAGGCGC
MpOrf4v2-S122A-3	GCGCCTTGTGCAAATCCAAGC
MpOrf4v2-R36A-5	CCAAGCCCAATGTGCGCGACTCATTGC
MpOrf4v2-R36A-3	GCAATGAGTCGCGCACATTGGGCTTGG
MpOrf4v2-R236A-5	GGTGACCATGCGGTTCCGCTG
MpOrf4v2-R236A-3	CAGCGGAACCGCATGGTCACC

Table S2: Plasmids used

Protein	Plasmid name	Parent Vector	Tag	MW (kDa)	Reference
CitA	pEMpOrf4v2	pET-24a	C-His6	30.7	This work
CitA-S122A	pEMpOrf4v2-S122A	pET-24a	C-His6	30.7	This work
CitA-R36A	pEMpOrf4v2-R36A	pET-24a	C-His6	30.7	This work
CitA-R236A	pEMpOrf4v2-R236A	pET-24a	C-His6	30.7	This work
PksCT SAT-KS-MAT	pEMpPksCT-SKM3	pET-24a	C-His6	144.0	[1]
PksCT PT	pEMpPksCT-PT	pET-24a	C-His6	41.5	[1]
PksCT ACP	pEMpPksCT-ACP	pET-24a	C-His6	15.0	[1]
PksCT CMeT	pEMpPksCT-CMeT	pET-24a	C-His6	46.6	[1]
PksCT CMeT-R	pEMpPksCT-CMeT-R	pET-24a	C-His6	92.7	[1]

Figure S1: Multiple sequence alignments of CitA with homologs

CitA was aligned to several putative hydrolases adjacent to other fungal Group VII NR-PKSs. Below are segments of the alignment showing the conserved catalytic residue S122 and the adjacent conserved basic residues R36 and R236. Please see references for more detail on AfoE, PkdA, PkeA, PkhA, and MpPKS5.[3,4,5] The alignment was performed in ClustalX v2.1.[6]

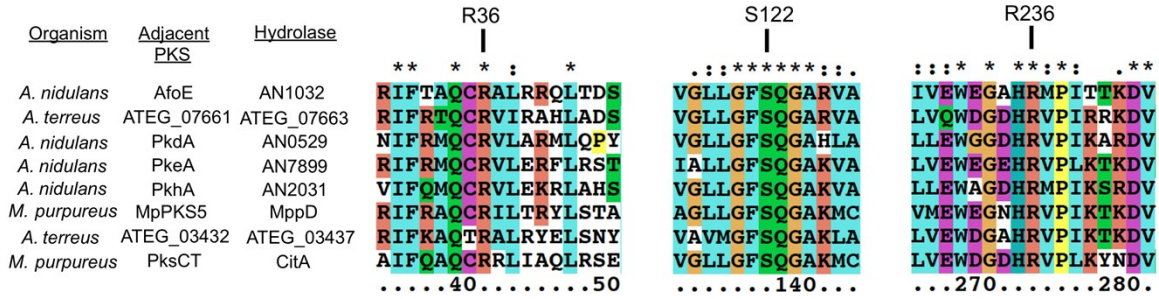


Figure S2: SDS-PAGE of PksCT ACP, CitA, and CitA mutants.

Separation of purified PksCT ACP and CitA by SDS-PAGE (16%) to show purity, with 5 µL per lane at 2 mg/mL total protein. M: BenchMark ladder (Invitrogen), with 50 and 20 kDa bands labeled, 1: CitA, 2: CitA-S122A, 3: CitA-R36A, 4: CitA-R236A, 5: PksCT ACP.

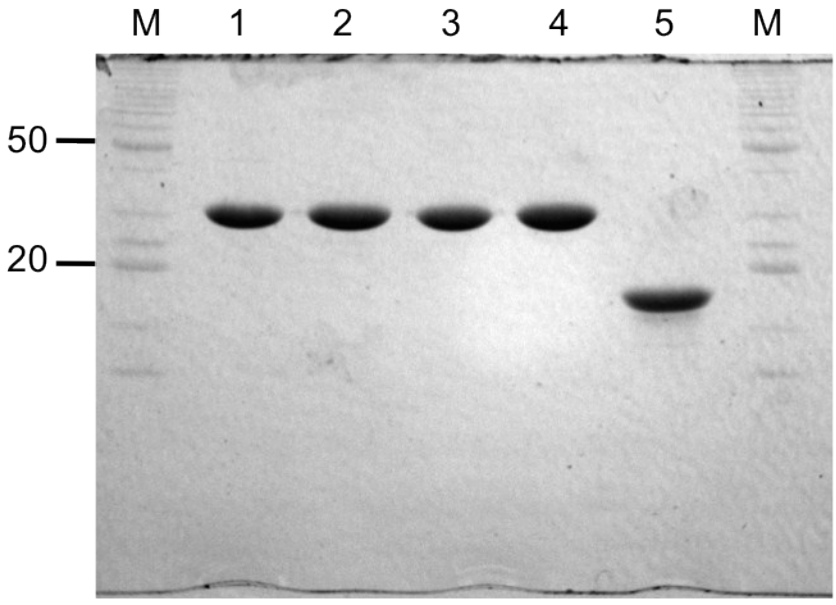


Figure S3: FPLC analysis of CitA and mutants.

Size exclusion separation of CitA and mutants by fast protein liquid chromatography (FPLC). Samples (1 mg) were applied to a Bio-Rad ENRich SEC 70 10 x 300 column, 0.5 mL/min, 100 mM KPO_4 pH 7.0, 5% glycerol (v/v), 4 °C, and absorbance monitored at 280 nm.

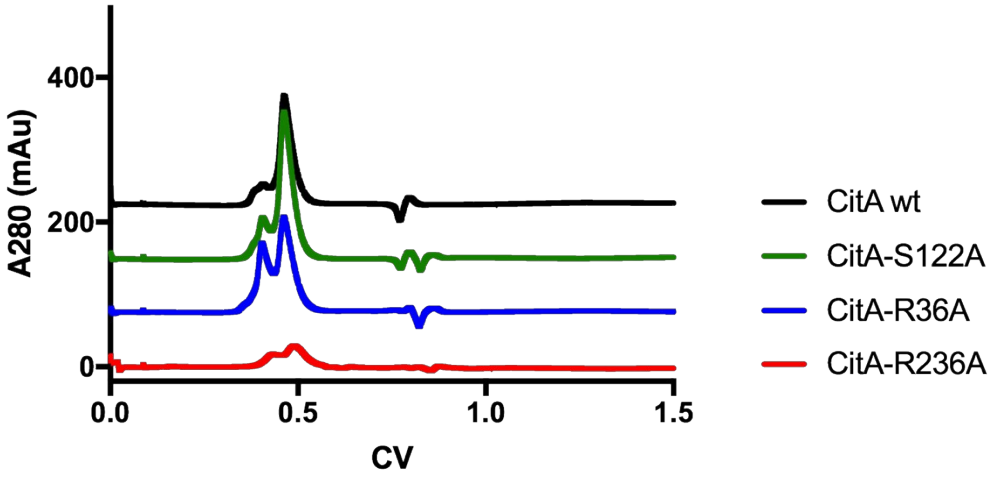


Figure S4: Circular dichroism spectra of CitA and mutants.

Spectra were acquired on an Aviv 420 CD spectrophotometer.

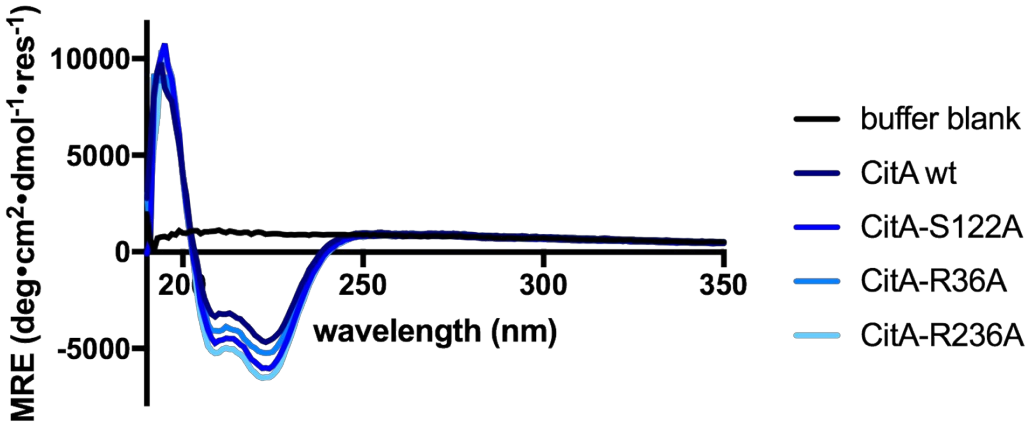
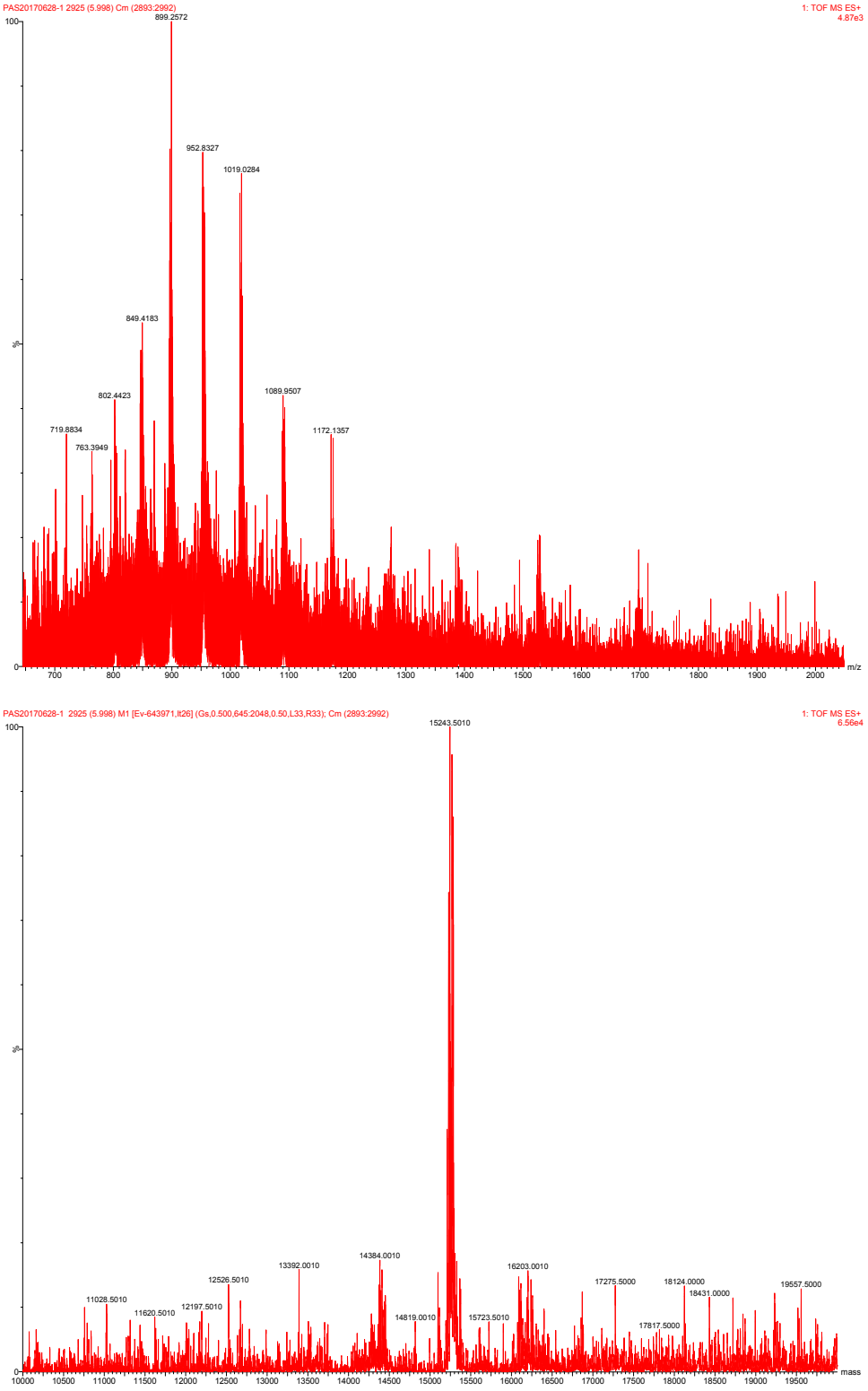
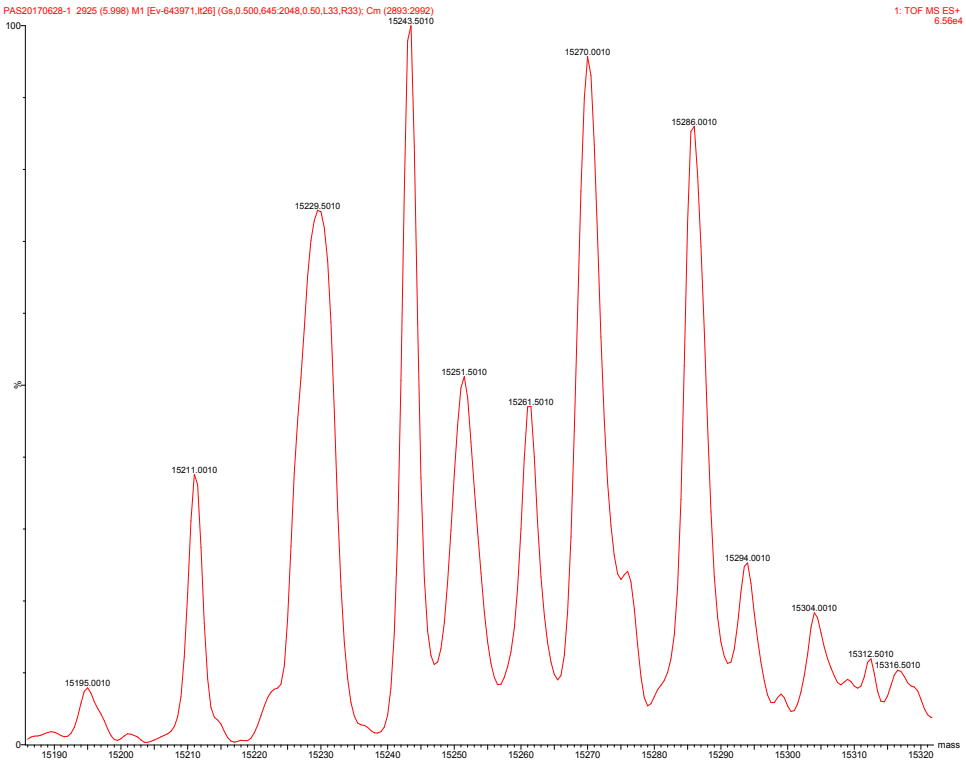


Figure S5: UPLC-ESI-MS data for *apo*-ACP$_{CT}$.

Detected ions (top), deconvoluted intact mass (middle), zoomed intact mass (bottom).

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100
%
0
15195.0010
15211.0010
15229.5010
15243.5010
15251.5010
15261.5010
15270.0010
15286.0010
15294.0010
15304.0010
15312.5010
15316.5010
15190
15200
15210
15220
15230
15240
15250
15260
15270
15280
15290
15300
15310
15320
mass

Figure S6: UPLC-ESI-MS data for Mal-*holo*-ACP$_{CT}$.

Detected ions (top) and deconvoluted intact mass (bottom).

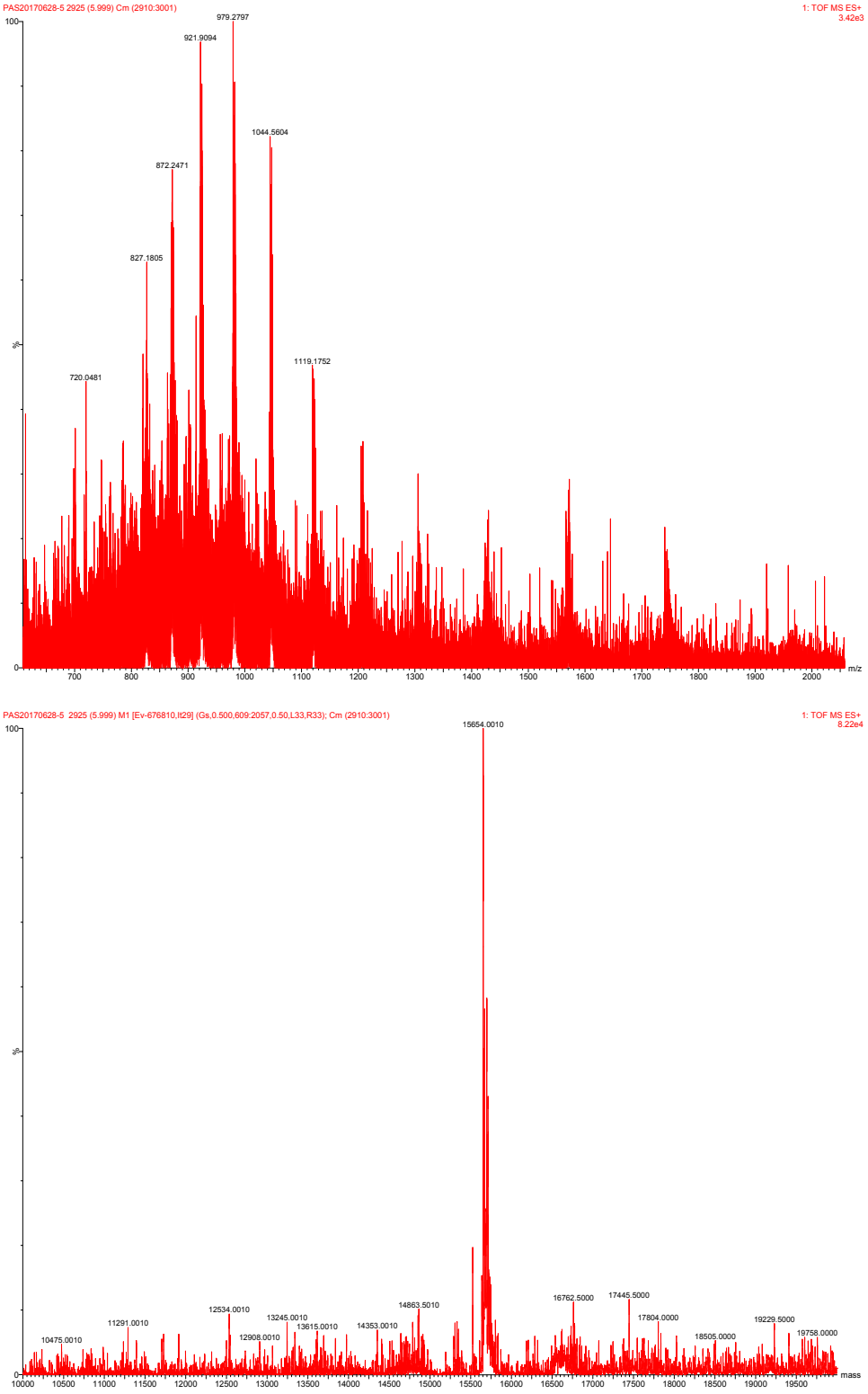


Figure S7: UPLC-ESI-MS data for Acac-*holo*-ACP$_{CT}$

Detected ions (top) and deconvoluted intact mass (bottom).

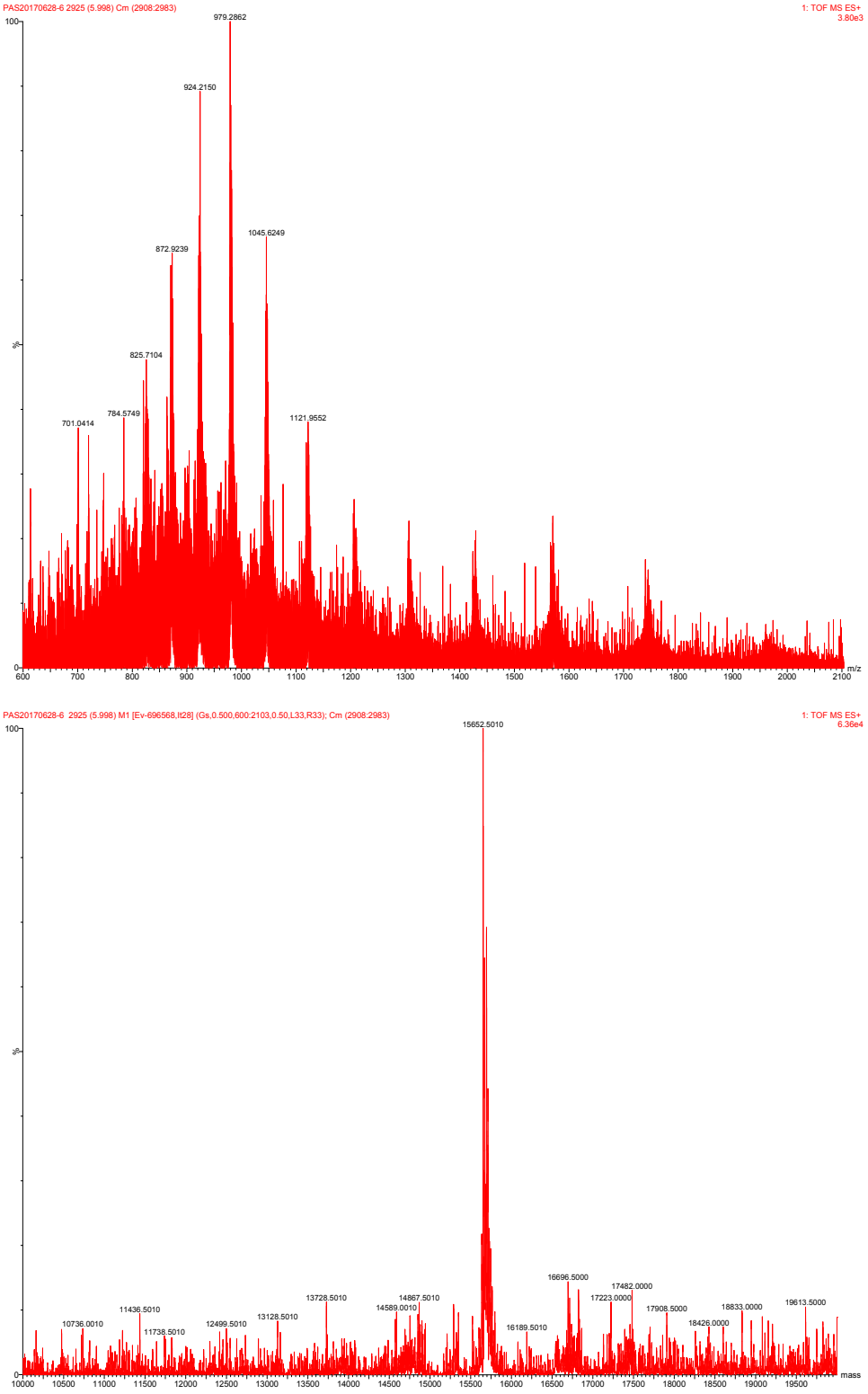


Figure S8: UPLC-ESI-MS data for Mal-*holo*-ACP$_{CT}$ + CitA

Detected ions (top) and deconvoluted intact mass (bottom).

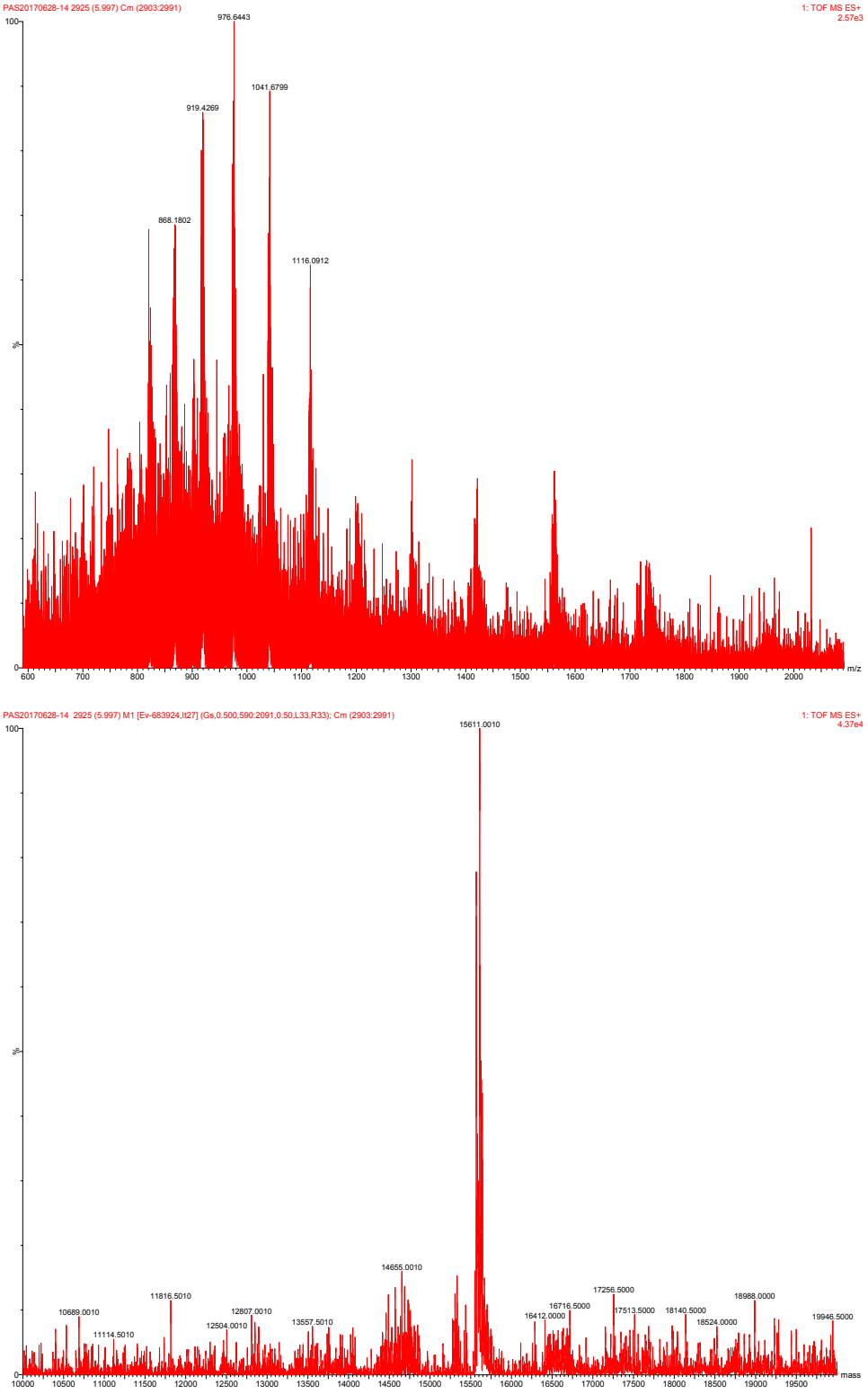


Figure S9: UPLC-ESI-MS data for Mal-*holo*-ACP$_{CT}$ + CitA

Detected ions (top) and deconvoluted intact mass (bottom).

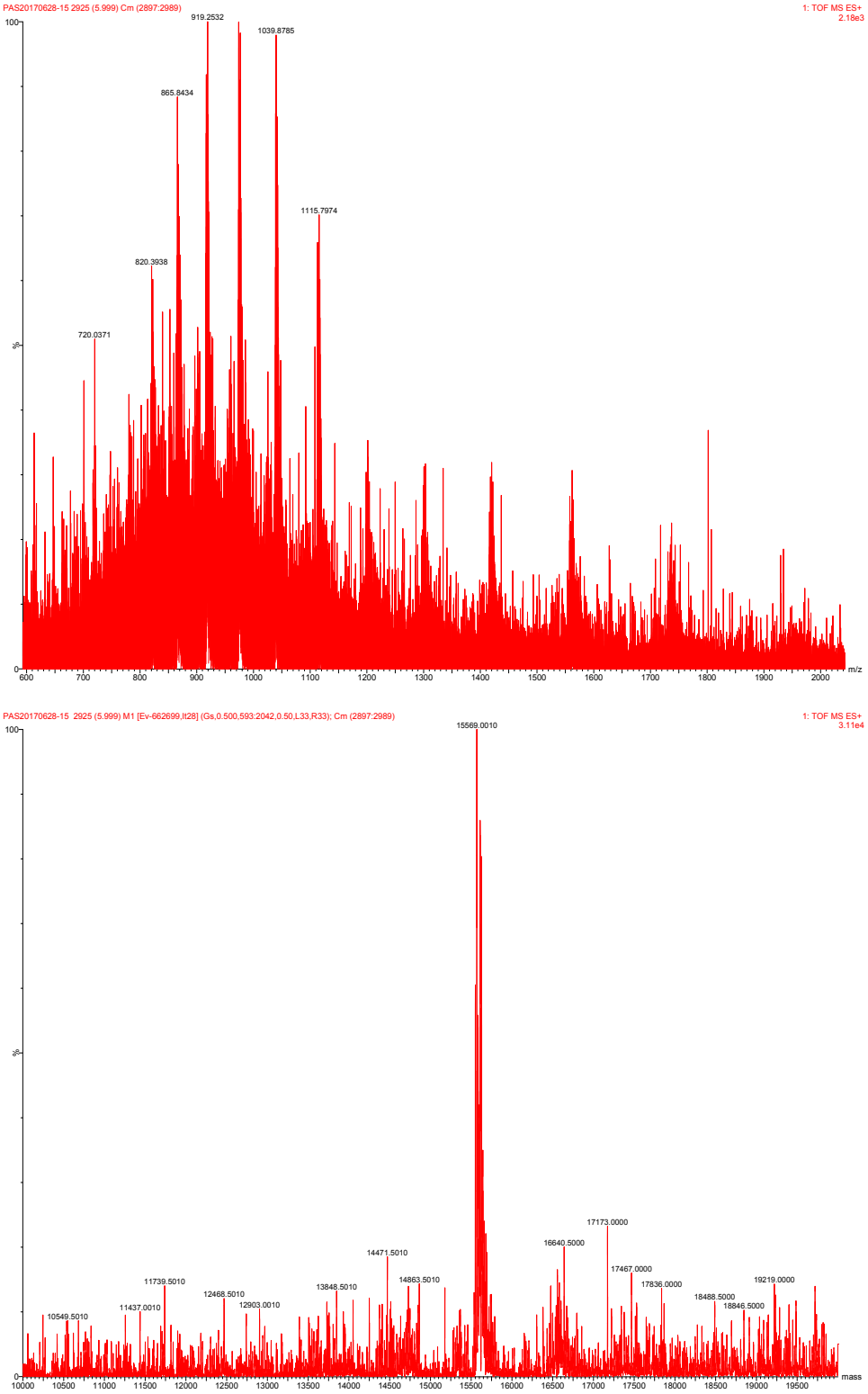


Fig S10: UPLC-ESI-MS traces for Mal-*holo*-ACP$_{CT}$/Acac-*holo*-ACP$_{CT}$ + CitA

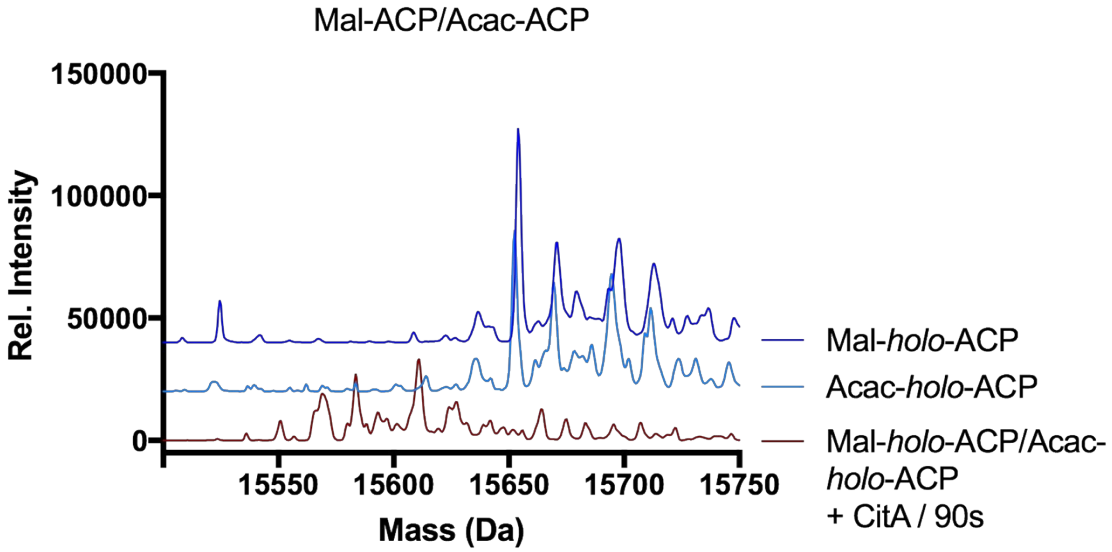
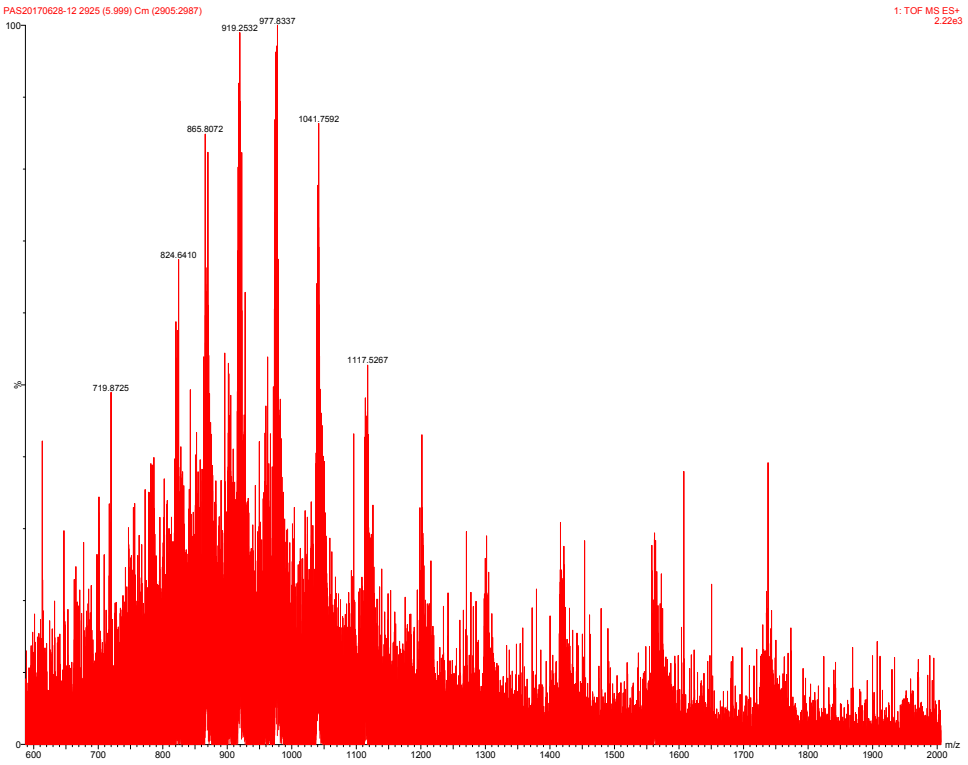
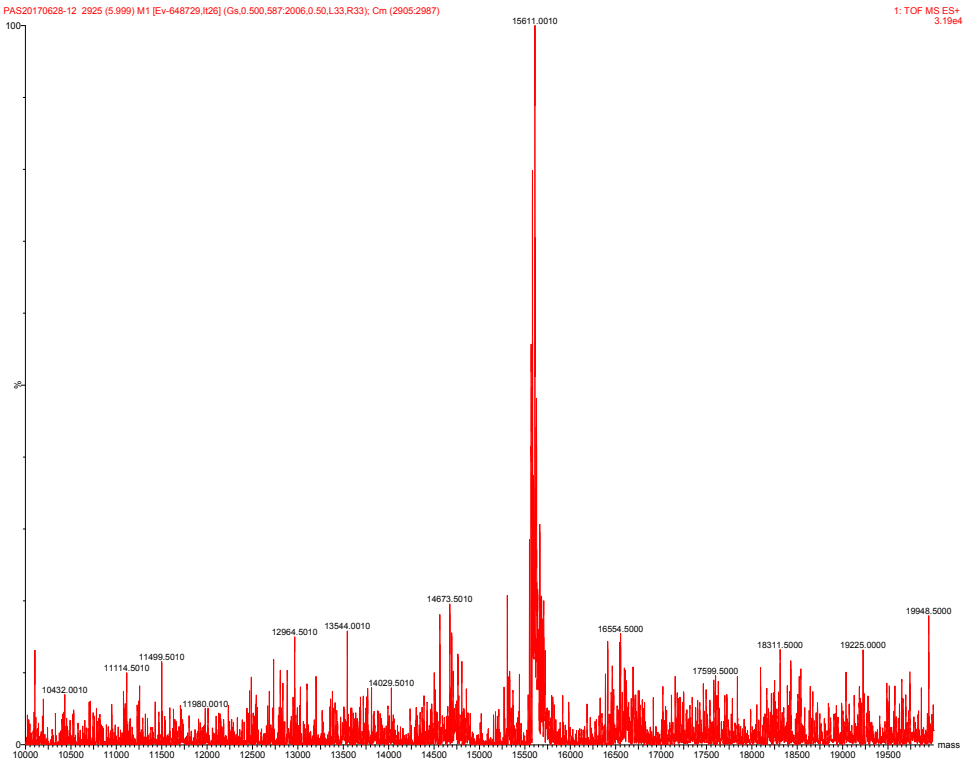


Fig S11: UPLC-ESI-MS data for Mal-*holo*-ACP$_{CT}$/Acac-*holo*-ACP$_{CT}$ + CitA

Detected ions (top), deconvoluted intact mass (middle), zoomed intact mass (bottom).

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3.19e4
100
%
0
15611.0010
14673.5010
19948.5000
13544.0010
12964.5010
16554.5000
18311.5000
19225.0000
11499.5010
11114.5010
17599.5000
14029.5010
10432.0010
11980.0010
10000 10500 11000 11500 12000 12500 13000 13500 14000 14500 15000 15500 16000 16500 17000 17500 18000 18500 19000 19500
mass

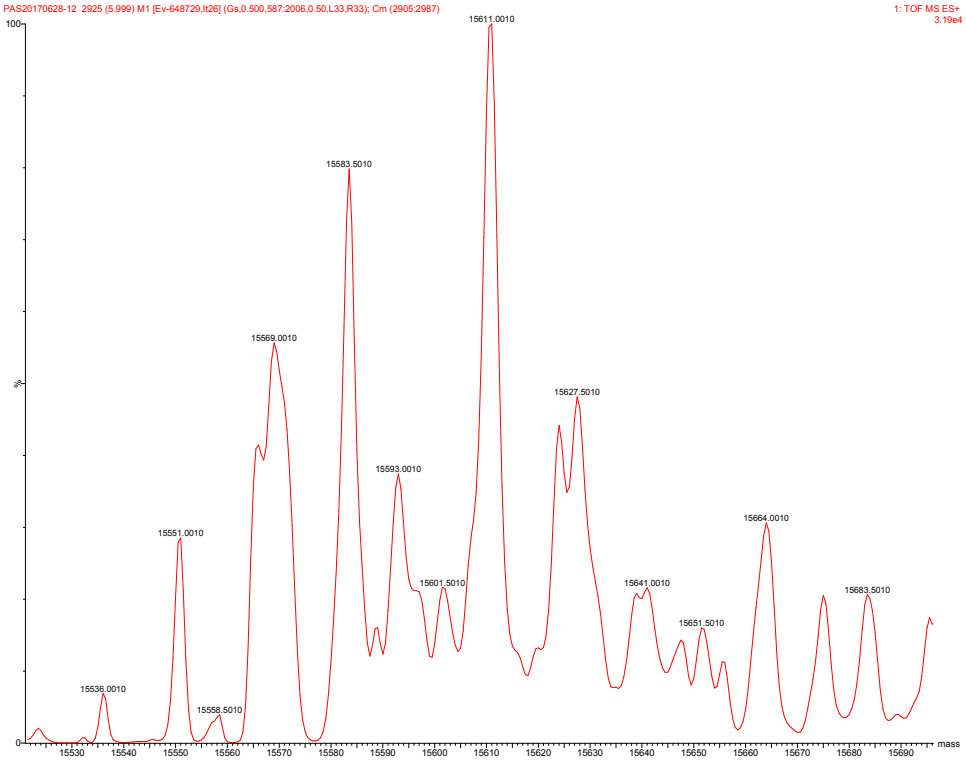
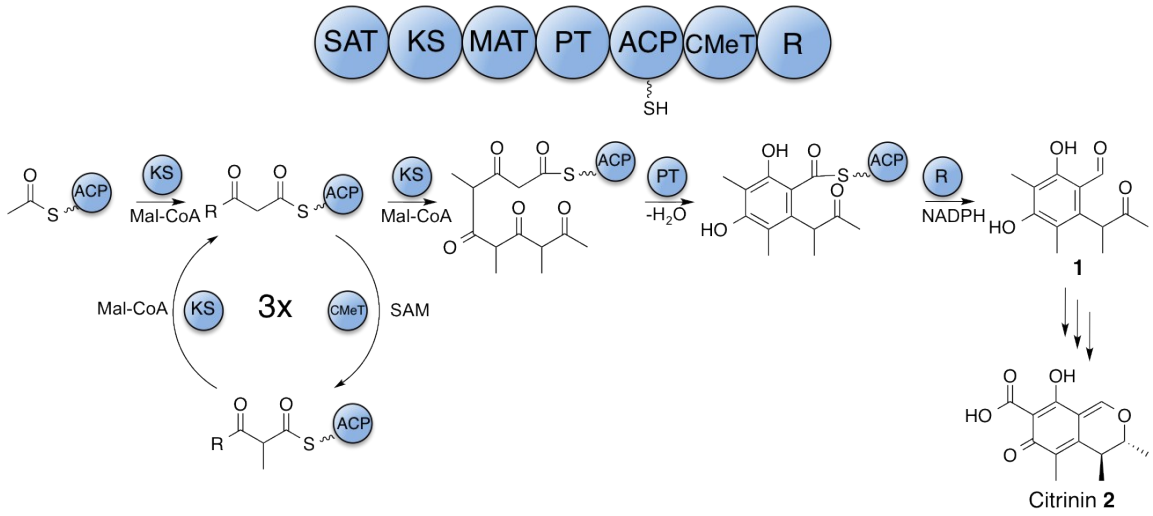

PAS20170628-12 2925 (5.999) M1 [Ev-648729,It26] (Gs,0.500,587:2006,0.50,L33,R33); Cm (2905:2987)
1: TOF MS ES+
3.19e4
100
%
0
15611.0010
15583.5010
15569.0010
15627.5010
15593.0010
15664.0010
15551.0010
15601.5010
15641.0010
15683.5010
15651.5010
15536.0010
15558.5010
15530 15540 15550 15560 15570 15580 15590 15600 15610 15620 15630 15640 15650 15660 15670 15680 15690
mass

Fig S12: Detailed schematic of ACP-bound intermediates in biosynthesis of 1 by PksCT.

The domain architecture of PksCT is shown (top). The *holo*-ACP is loaded with an acetyl starter unit and then subjected to three rounds of iterative extension and methylation by the KS and CMeT domains, before a final round of extension. The intermediate is then cyclized to give an aromatic acyl-*holo*-ACP intermediate that is reductively released to **1**.



References

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