Electronic Supplementary Material (ESI) for Molecular BioSystems

A photocrosslinking assay for reporting protein interactions in polyketide and fatty acid synthases

Zhixia Ye, Morgan Bair, Hemant Desai and Gavin J. Williams

Electronic Supplementary Information

Cloning of ACP2 and AcpP

ACP2 gene was synthesized and codon optimized by Blue Heron Biotechnology, Inc (Bothell, WA). The ACP2 gene was then subcloned into pET28a using *Nde*I and *Eco*RI restriction sites. Briefly, the digested ACP2 containing plasmid was run on a 1% agarose gel stained with 0.5 μg/ml ethidium bromide. The ACP2 gene was excised and purified with the QIAquick Gel Extraction Kit (QIAgen). Inserts were digested with 20 units each of *Nde*I and *Eco*RI (NEB) at 37 °C for 4 h, followed by purification with the gel extraction kit. Inserts were ligated into pET28a that had been digested with the same restriction enzymes. The resulting ligation product mixture was transformed into *E. coli* DH5a competent cells and plated onto LB/Kan plates. Colonies were screened by restriction analysis and DNA sequencing (performed by GeneWiz, Inc., South Plainfield, NJ).

The gene for AcpP was PCR amplified from genomic DNA of *E. coli* BL21 using primers AcpP-NdeI-FOR and AcpP-EcoRI-REV (see Table 1). PCR reaction mixture contained primers at 2 μM, 0.2 mM dNTP mix, 20 ng template plasmid, 1 unit Phire Hot Start DNA polymerase (Thermo Scientific, Waltham, MA), in buffer supplied by the manufacturer.

Thermocycling conditions were as follows:

1. 98 °C for 3 min
2. 30 cycles of: 98 °C for 20 sec, 60 °C for 1 min, 72 °C for 1 min/kb
3. 72 °C for 10 min

AcpP was then cloned into pET28a via *Nde*I and *Eco*RI restriction sites using the same procedure as for ACP2.

Cloning and expression of KSs

Type II fatty acid synthase KS domains FabF, FabB and FabH were each PCR amplified from genomic DNA of *E. coli* BL21 (see Table 1 for primer sequences). Genomic DNA was prepared by growing an overnight culture of BL21(DE3) in 3 ml LB and resuspending the bacterial cells in 100 μl sterile water, followed by boiling at 100 °C for 5 min. The sample was centrifuged and aliquots used for PCR. Each FabB and FabH were cloned into pET28a via *Bam*HI and *Sac*I sites, while FabF was cloned into pETDuet via *Hind*III and *Not*I sites. Cloning procedure was similar to that described for ACP2. Protein expression of FabF[4], FabB[4] and FabH[5] was accomplished by following published procedures.[1, 2]

Unnatural amino acid incorporation

Amino acid pBpa was incorporated into ACP2 and AcpP using *Methanocaldococcus jannaschii* aminoacyl-tRNA synthetase(s) (aaRS)/suppressor tRNA pairs developed by the Schultz group,[3] in response to the amber stop codon, TAG. Both the WT (ACP2 and AcpP) and mutant (ACP2-pBpa and AcpP-pBpa) carrier proteins were expressed following published procedures.[4, 5]

Site-directed mutagenesis

Alanine scanning mutants of FabF were constructed using the QuickChange Site-Directed Mutagenesis Kit from Stratagene, according to the manufacturer. Oligonucleotides used are shown in Table 1.

Table 1: Primers used in this study.

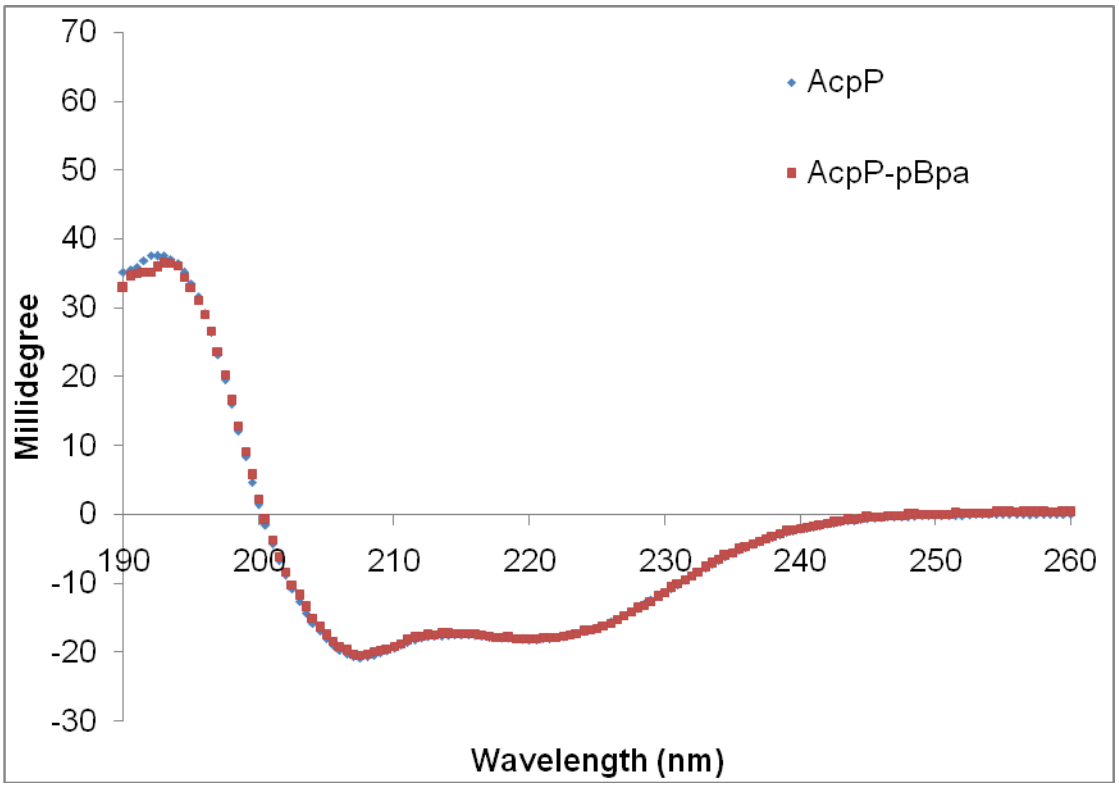
Primer name	Primer sequence
AcpP-NdeI-FOR	AGCTTCCATATGATGAGCACTATCGAAGAACGCG
AcpP-EcoRI-REV	AGCTTCGAATTCTTACGCCTGGTGGCCGTTGATG
AcpP-Ser36TAG-FOR	CTGGGCGCGGATTAGCTTGACACCGTTGAG
AcpP-Ser36TAG-REV	CTCAACGGTGTCAAGCTAATCCGCGCCCAG
ACP2-Ser54TAG-FOR	TTAGGCTTTGACTAGTTAGCTGCCGTACGTC
ACP2-Ser54TAG-REV	GACGTACGGCAGCTAACTAGTCAAAGCCTAA
ACP6-NdeI-FOR	TGCAGCTTCCATATGGCGGCCCCGGCGCGG
ACP6-EcoRI-REV	TGCAGCTTCGAATTCTTAGAGCTGCTGTCCTAT
ACP6-S45TAG-FOR	CTCGGCTTCGACTAGCTGACCGCGGTCG
ACP6-S45TAG-REV	CGACCGCGGTCAGCTAGTCGAAGCCGAG
FabF-HindIII-FOR	AGCTTCAAGCTT GTGTCTAAGCGTCGTGTAGTTG
FabF-NotI-REV	AGCTTCGCGGCCGCTTAGATCTTTTTAAAGATCAAAGAACCATT AGTGCC
FabB-BamHI-FOR	AGCTTCGGATCCATGAAACGTGCAGTGATTACTGG
FabB-SacI-REV	AGCTTCGAGCTCTTAATCTTTCAGCTTGCGCATTACC
FabH-BamHI-FOR	AGCTTCGGATCC ATGTATACGAAGATTATTGGTACTGGCAGC
FabH-SacI-REV	AGCTTCGAGCTCTTAGAAACGAACCAGCGCGGAGC
FabF-N57A-FOR	GGCTTAGTAAAGGATTTT*GCG*TGTGAGGACATTATCTCGCGC
FabF-N57A-REV	GCGCGAGATAATGTCCTCACA*CGC*AAAATCCTTTACTAAGCC
FabF-I92A-FOR	GATTCTGGCCTTGAA*GCG*ACGGAAGAGAACGCAAC
FabF-I92A-REV	GTTGCGTTCTCTTCCGT*CGC*TTCAAGGCCAGAATC
FabF-R206A-FOR	GTTTTGGCGCGGCA*GCG*GCATTATCTACC
FabF-R206A-REV	GGTAGATAATGC*CGC*TGCCGCGCCAAAAC
FabF-L208A-FOR	GCGGCACGTGCA*GCG*TCTACCCGCAATG
FabF-L208A-REV	CATTGCGGGTAGA*CGC*TGCACGTGCCGC
FabF-E225A-FOR	GCCCGTGGGATAAA*GCG*CGTGATGGTTTCGTACTG
FabF-E225A-REV	CAGTACGAAACCATCACG*CGC*TTTATCCCACGGGC
FabF-D227A-FOR	GGGATAAAGAGCGT*GCG*GGTTTCGTACTGGGCG
FabF-D227A-REV	CGCCCAGTACGAAACC*CGC*ACGCTCTTTATCCC
FabF-T307A-FOR	CACGGTACTTCT*GCG*CCGGCTGGCGATAAAG
FabF-T307A-REV	CTTTATCGCCAGCCGG*CGC*AGAAGTACCGTG

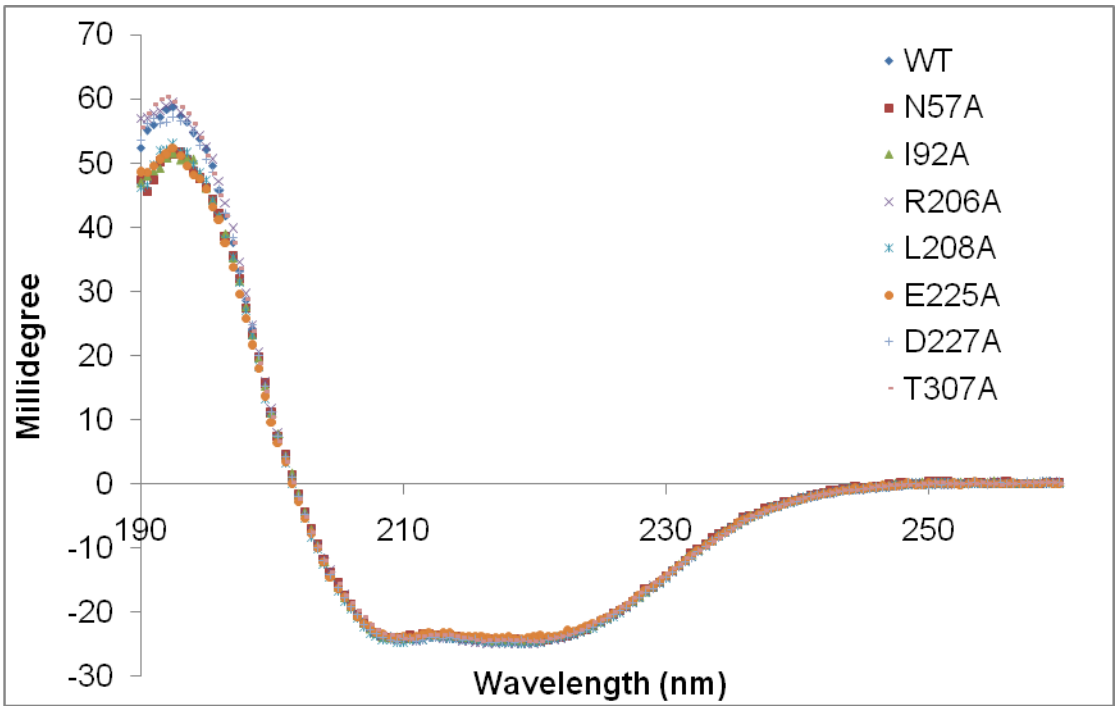
Purification of expressed proteins

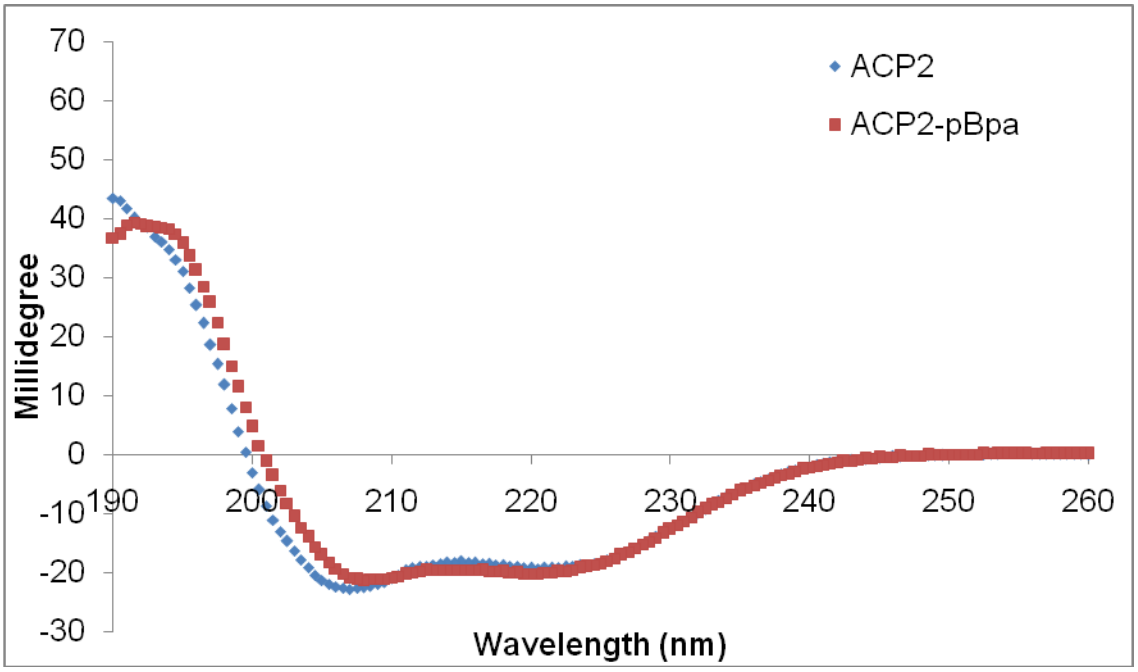
Cells were collected by centrifugation at 5,000 *g* for 20 min, and resuspended in 20 mL of 100 mM Tris-HCl pH 8.0 containing 300 mM NaCl and then lysed by sonication. Following centrifugation at 10,000 *g*, the soluble extract was loaded onto a 1 mL HisTrap HP column (GE Healthcare, Piscataway, NJ) and purified by fast protein liquid chromatography using the following buffers: wash buffer [20 mM phosphate (pH 7.4) containing 0.5 M NaCI and 20 mM imidazole] and elution buffer [20 mM phosphate (pH 7.4) containing 0.5 M NaCI and 200 mM imidazole]. The purified protein was concentrated using an Amicon Ultra 10,000 MWCO centrifugal filter (Millipore Corp., Billerica, MA) and stored as 10% glycerol stocks at -80 °C. Protein purity was verified by SDS-PAGE. Protein quantification was carried out using the Bradford Protein Assay Kit from Bio-Rad.

CD spectroscopy of AcpP, ACP2, and FabF mutants

CD measurements were performed with a JASCO 810 CD Spectropolarimeter. Samples for CD were buffer exchanged into CD buffer (10 mM potassium phosphate, 50 mM sodium sulfate, pH 7.4) and the concentration of samples was 0.2 mg/ml. Spectra from 190 nm to 260 nm were scanned at a step of 0.5 nm at 20 ℃ in a 0.1 cm cuvette, with ten repeats. The scan speed was 100 nm/min.







Photocrosslinking conditions

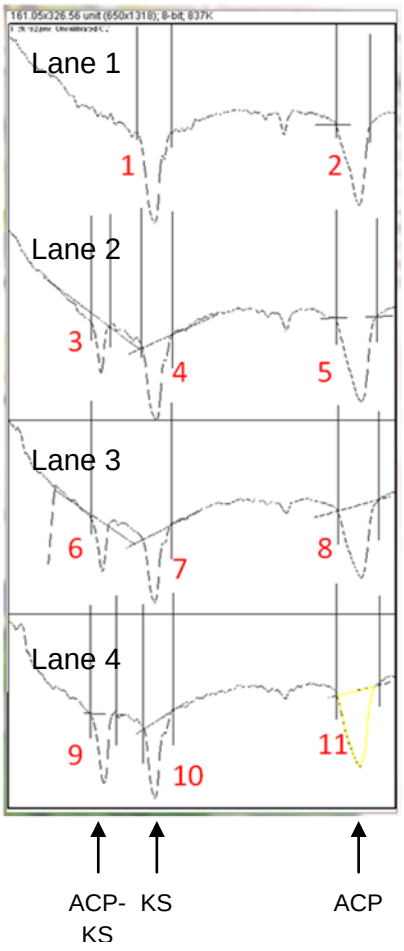
Photocrosslinking reactions were carried out in wells of a 96-well microtiter plate on ice. Reaction volume was 50 µL in PBS buffer, ACPs were about five fold in excess of KSs (8.8 µM). Reaction was irradiated at 365 nm with a handheld UV lamp (transilluminator, 6W) and then analyzed by SDS-PAGE (4-12% gradient).

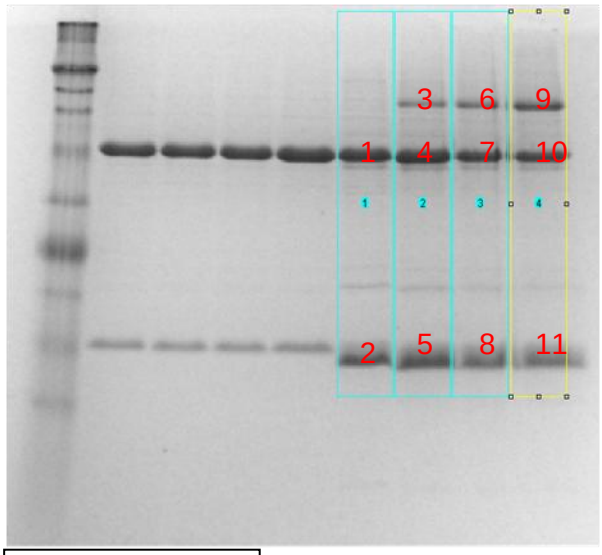
Densitometric analysis of photocrosslinking

Photocrosslinking efficiency was expressed as percentage conversion from KS (this was the limiting reagent) using the following formula:

% crosslinking = (peak Area) $_{ACP\text{-}KS}$ / [(Peak Area)$_{KS}$ + (Peak Area)$_{ACP\text{-}KS}$]

Peak areas were determined by densitometry using ImageJ,[6] an example is shown below:





Peak 1 = 234
Peak 2 = 237
Peak 3 = 71
Peak 4 = 196
Peak 5 = 306
Peak 6 = 69
Peak 7 = 142
Peak 8 = 257
Peak 9 = 116
Peak 10 = 155
Peak 11 = 271

e.g. Lane 4:

% crosslinking =
116/(155+116) = 43%

Mass Spec analysis

The protein samples for MS were exchanged into 100 mM ammonium acetate with Zeba-spin desalting columns (Pierce Protein Research Products, Rockford, IL) before being submitted for MS analysis on an Agilent Technologies 6210 LC-TOF instrument. For AcpP-pBpa and ACP2-pBpa, samples were injected by direct infusion. For photocrosslinked AcpP-FabF, protein was injected through a Poroshell 300SB-C18, Analytical 2.1*75 mm, 5 Micron (Agilent). Although salt adducts were common, proteins a natural amino acid at position 36 and 50 of AcpP or ACP2 respectively (small molecular weight), were not identified.

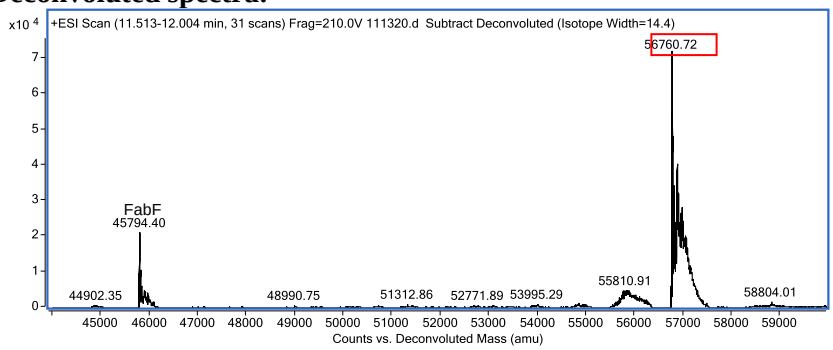
Table 2: Mass spectrometry analysis of WT and mutant proteins.

Protein	Calculated mass[a] (Da)	Experimental mass (Da)	Δ (Da)
AcpP	10803.306	10802.75	-0.56
AcpP-S36pBpa	10966.48	10967.13	+0.65
ACP2-S50pBpa	20521.73	20521.89	+0.16
AcpP-FabF	56761.20	56760.72	-0.48

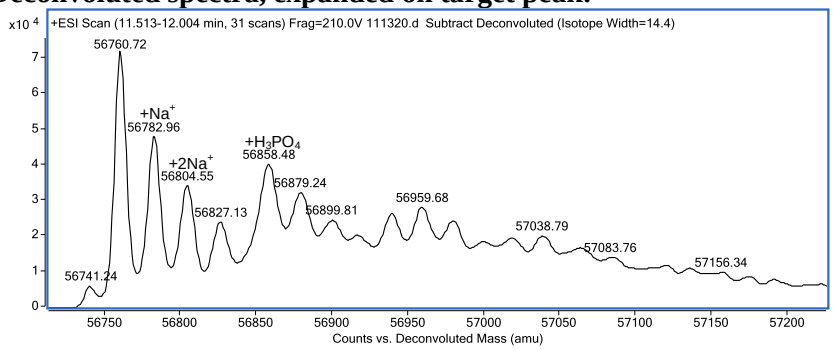
[a]The calculated mass is adjusted to include loss of N-terminal methionine (-130.194 Da).

A) Photocrosslinked AcpP-FabF

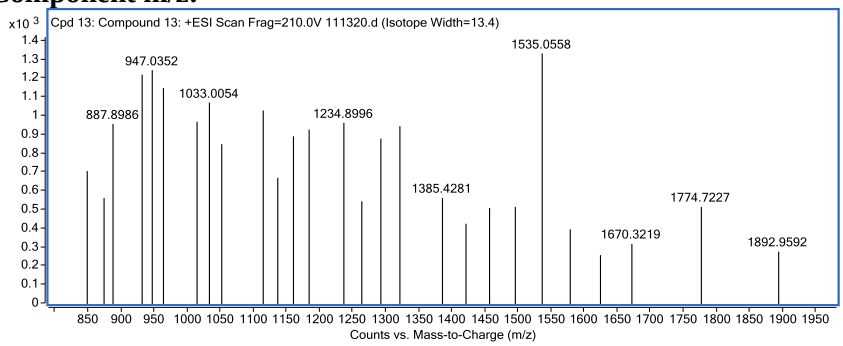
Deconvoluted spectra:



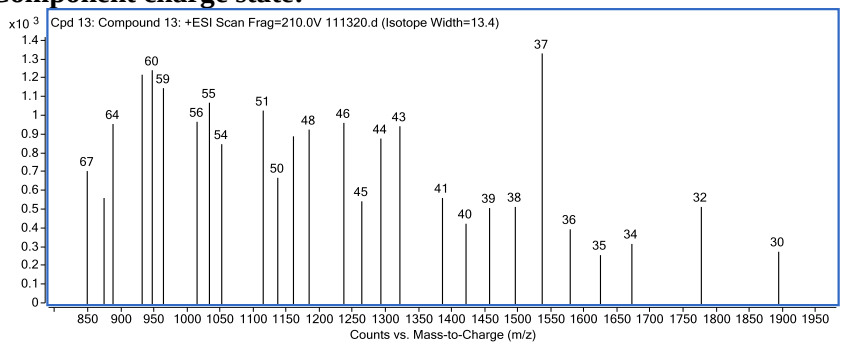
Deconvoluted spectra, expanded on target peak:



Component m/z:

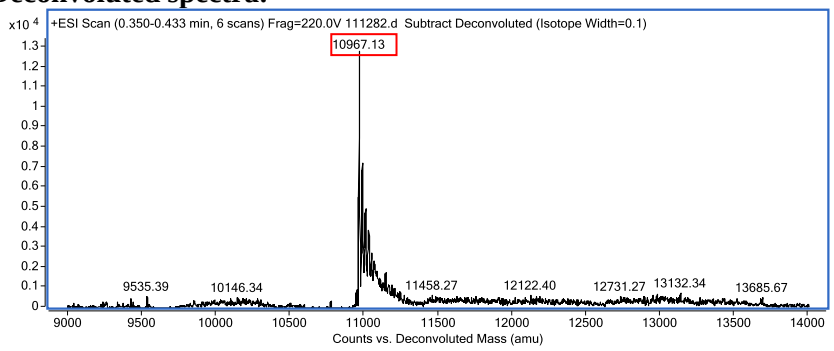


Component charge state:

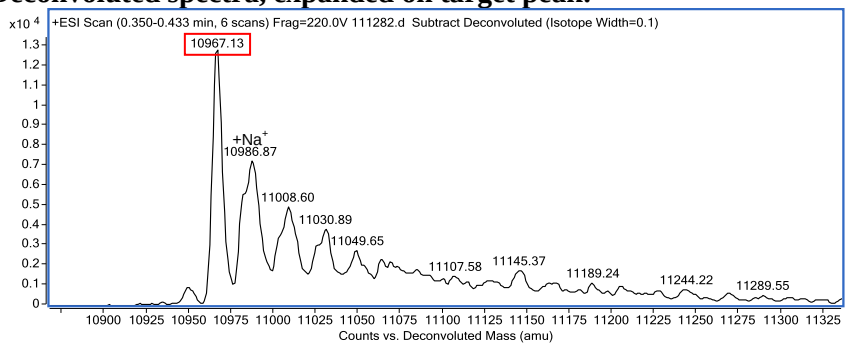


B) AcpP-pBpa

Deconvoluted spectra:



Deconvoluted spectra, expanded on target peak:



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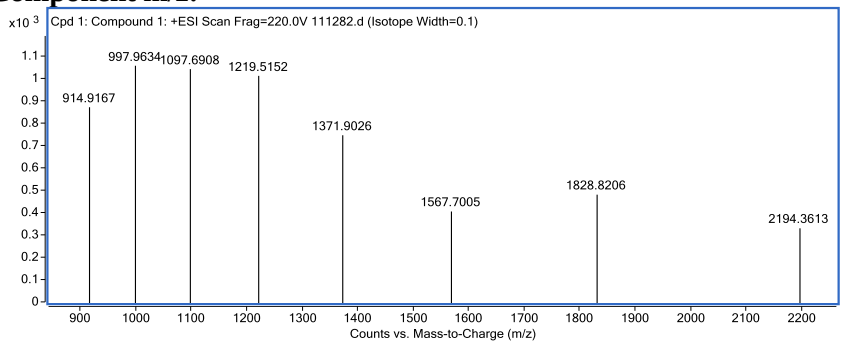

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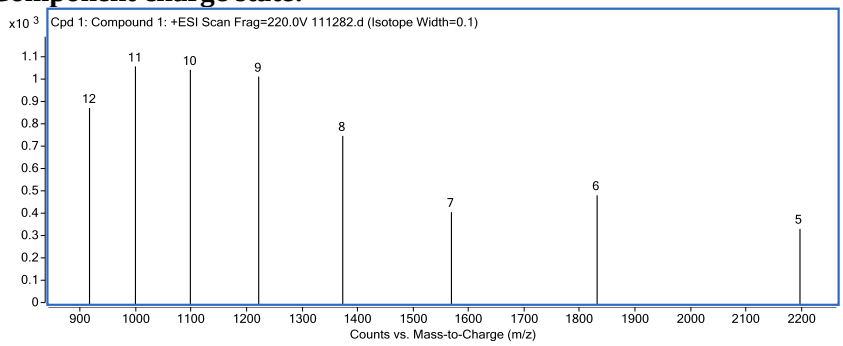
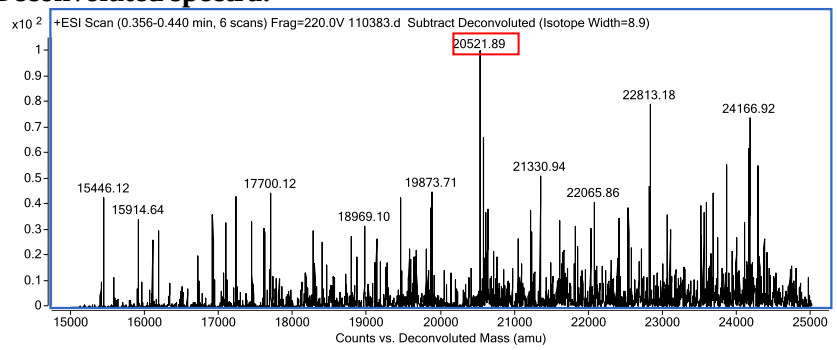
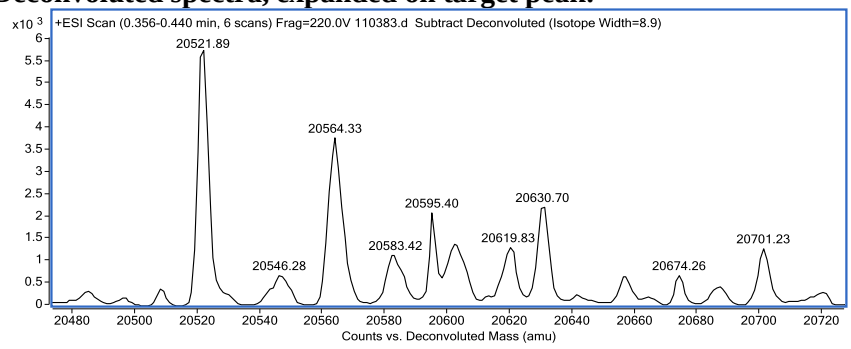


C) ACP2 pBpa

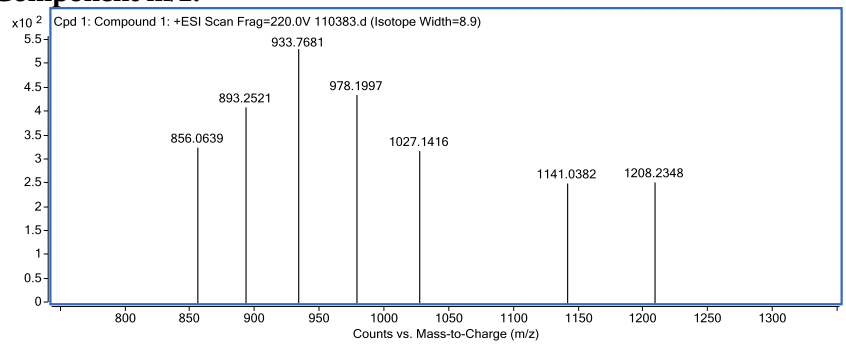
Deconvoluted spectra:



Deconvoluted spectra, expanded on target peak:



Component m/z:



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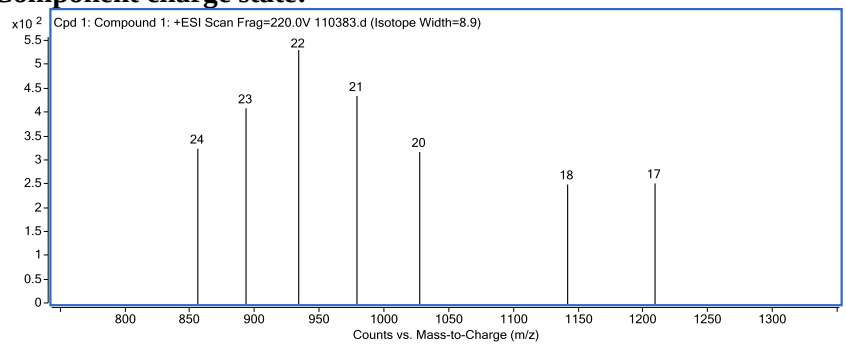

References

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