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Top-down mass spectrometry on low-resolution instruments: Characterization of phosphopantetheinylated carrier domains in polyketide and non-ribosomal biosynthetic pathways

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Abstract—Mass spectrometry (MS) is an important tool for studying non-ribosomal peptide, polyketide, and fatty acid biosynthesis. Here we describe a new approach using multi-stage tandem MS on a common ion trap instrument to obtain high-resolution measurements of the masses of substrates and intermediates bound to phosphopantetheinylated (*holo*) carrier proteins. In particular, we report the chemical formulas of 12 diagnostic MS^3 fragments of the phosphopantetheine moiety ejected from *holo* carrier proteins during MS^2. We demonstrate our method by observing the formation of *holo*-AcpC, a putative acyl carrier protein from *Streptococcus agalactiae*.
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The biosynthesis of polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) natural products, including the well-established therapeutic agents penicillin, vancomycin, and rapamycin, requires post-translational modification of carrier proteins by addition of a 4′-phosphopantetheine (PPant) arm. The sulfur at the free end of this moiety forms a thioester bond with substrates and intermediates at each step of the biosynthetic process. Because these substrates and intermediates increase the overall mass of the associated carrier proteins, the latter are ideal targets for 'top-down' characterization of PKS and NRPS pathways by mass spectrometry (MS).[1] A top-down approach was recently shown to facilitate the study of phosphopantetheinyl-tethered substrates.[2] Thermal activation by infrared multiphoton dissociation (IRMPD) or collision-induced dissociation (CID) causes the *holo* form of acyl and peptidyl carrier proteins, which are found in PKS and NRPS pathways, respectively, to consistently 'eject' their PPant arm, preserving the thioester linkage to the substrate (Fig. 1a). This 'PPant ejection assay' allows the mass of substrates loaded onto carrier proteins to be readily deduced from the mass of the corresponding PPant fragments.[3] Specifically, when no substrate is linked to the PPant arm, the fragment ejected from the carrier protein has chemical formula $C_{11}H_{21}N_2O_3S^+$, giving an MS^2 peak at *m/z* 261.1267. When an acyl or peptidyl substrate is bound to the PPant arm, the PPant peak in the MS^2 spectrum is shifted by an amount equal to the mass of the substrate less a water molecule.[4]

The PPant ejection assay can greatly facilitate the characterization of NRPS and PKS systems by mass spectrometry, but demands instruments capable of both high sensitivity and high accuracy. Indeed, PPant ejection was originally observed on a custom-built Fourier transform ion cyclotron resonance (FTICR)-MS that utilized an accumulation octopole to collect ions before transmission to the FTICR mass analyzer.[5] However, commercial FTICR-MS instruments may not produce peaks sufficiently intense for reliable detection of the ejected PPant species. Conversely, ion trap mass

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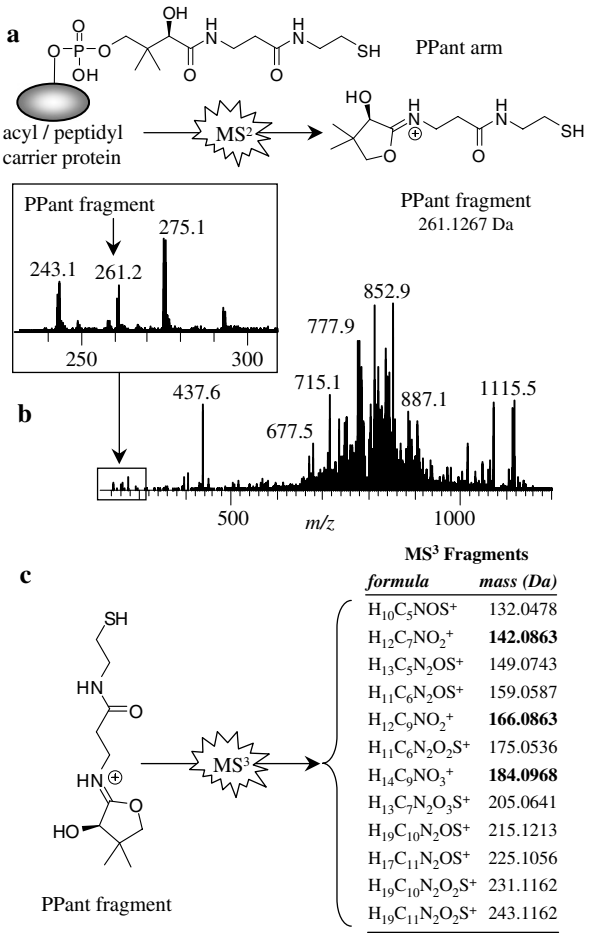

Figure 1. The MS^3 phosphopantetheine fragmentation method. (a) MS^2 experiments on *holo*-carrier proteins result in phosphopantetheine (PPant) ejection.[2] (b) MS^2 on the predicted 16+ charge state (*m*/*z* 796) of *holo*-AcpC produced a peak at *m*/*z* 261.2, which is consistent with the PPant fragment. (c) MS^3 on this PPant fragment gave 12 peaks, which may be useful to confirm the mass of a substrate linked to the PPant arm. The masses shown in bold should be substrate-independent, because the corresponding proposed formulas do not include the sulfur atom necessary to bind the substrates.

spectrometers, while typically more sensitive and capable of higher scan rates than FTICR instruments, do not always provide sufficient accuracy and signal-to-noise ratio to confirm the elemental composition of the PPant ejection peak.

These limitations became evident when we applied the PPant ejection assay on a Finnigan LTQ MS to characterize AcpC (Accession No. NP_735098), a putative 12.4-kDa acyl carrier protein encoded by the fourth open-reading frame in the *cyl* gene cluster of the bacterial pathogen *Streptococcus agalactiae*, or group B *Streptococcus* (GBS).[6] The *cyl* gene cluster of GBS is involved in the production of the β-hemolytic/cytolytic activity that contributes to the virulence of GBS, a major cause of infant mortality in industrialized countries. Remarkably, the structure of the final molecule produced by the *cyl* gene cluster is still unknown.[7] Because disruption of the *acpC* gene by transposon mutagenesis has suggested that AcpC is essential for hemolytic phenotype of GBS,[6] we decided to study this protein. To begin our investigation, we expressed an AcpC-His_6 fusion construct in *Escherichia coli* and enriched the protein by Ni–NTA affinity chromatography.[16] The resulting sample was subjected to nano-electrospray ionization (ESI) and collision-induced dissociation (CID) of the predicted 16+ charge state (*m*/*z* 796) in the linear ion trap, yielding a clearly discernible peak at *m*/*z* 261.2 (Fig. 1b).[17] This peak matches the mass of the MS^2 PPant fragment, suggesting that AcpC is a carrier protein and that AcpC can be phosphopantetheinylated by a PPant transferase (PPTase) naturally occurring in *E. coli*. However, the peak at *m*/*z* 261.2 was much weaker than the other peaks present in the MS^2 spectrum and the accuracy of the ion trap mass analyzer was not sufficient for unequivocal assignment of this peak to the PPant fragment.

We therefore investigated whether subjecting the putative MS^2 PPant fragment of *holo*-AcpC to another round of fragmentation would yield a more informative mass spectrum. Indeed, we observed a rich pattern of MS^3 peaks that appeared to be uniquely associated with the ejected PPant arm (Fig. 1c).[18] We reasoned that this fragmentation pattern could be generally exploited as a diagnostic tool to detect the presence of carrier proteins bearing the PPant arm and to determine the mass of any PPant-linked species. Hence, we developed an alternative top-down method for the unambiguous characterization of substrates and intermediates bound to carrier proteins of PKS and NRPS biosynthetic pathways.

The likely molecular formulas for 12 of the observed MS^3 PPant ions (Fig. 1c) indicate that nine of the fragments, whose calculated *m*/*z* values are 132.0, 149.1, 159.1, 175.1, 205.1, 215.1, 225.1, 231.1, and 243.1, contain the PPant thiol group, which bears the substrate during the biosynthesis of natural products. Loading a substrate onto the PPant arm would then be expected to shift these thiol-containing MS^3 fragments to higher masses, but should leave the mass of the other three MS^3 fragments unchanged at the calculated *m*/*z* values of 142.1, 166.1, and 184.1. The latter MS^3 ions provide a diagnostic pattern for detecting PPant ejection, while the nine thiol-containing MS^3 ions can be used to infer the mass of the loaded substrate. The *m*/*z* value of the MS^2 PPant peak resulting from fragmentation of a given carrier protein, as well as the *m*/*z* values of the MS^3 peaks resulting from fragmentation of the PPant fragment itself, can all be calculated a priori from the theoretical masses listed in Figure 1c and from the mass of the given substrate.[8]

To evaluate our approach and to optimize the instrumental settings, CouN5, a 11.8-kDa peptidyl carrier protein from the coumermycin A_1 NRPS biosynthetic pathway of *Streptomyces rishiriensis* DSM 40489,[9] was used as a model system, because CouN5 is known to produce an abundant MS^2 PPant fragment ion. *Holo*-CouN5 was prepared by incubation of *apo*-CouN5[19] with CoA and Sfp (Accession No. P39135), a 26.1-kDa promiscuous PPTase from the surfactin

biosynthetic pathway of *Bacillus subtilis*.[10] The reaction products were ionized by nano-capillary liquid chromatography (LC)-ESI and analyzed with the linear ion trap. The predicted 15+ charge state (*m/z* 807) of *holo*-CouN5 and the PPant ion (*m/z* 261.1) were sequentially subjected to CID, resulting in MS^3 peaks at *m/z* values of 132.1, 142.1, 149.0, 159.1, 166.1, 175.0, 184.1, 205.3, 215.0, 225.1, 231.2, and 243.2 (Fig. 2a).[20] These values agree with those observed for AcpC (Fig. 1c). We verified the expected substrate-dependent mass shifts by performing MS^3 experiments on CouN5 loaded with acetamide by Sfp, and by observing the expected +57-Da shift of the nine thiol-containing fragments (Fig. 2b).[21] Since a total of 12 MS^3 PPant fragments must be present at *m/z* values predictable from the mass of the PPant-linked substrate, this mass can be determined with high confidence.

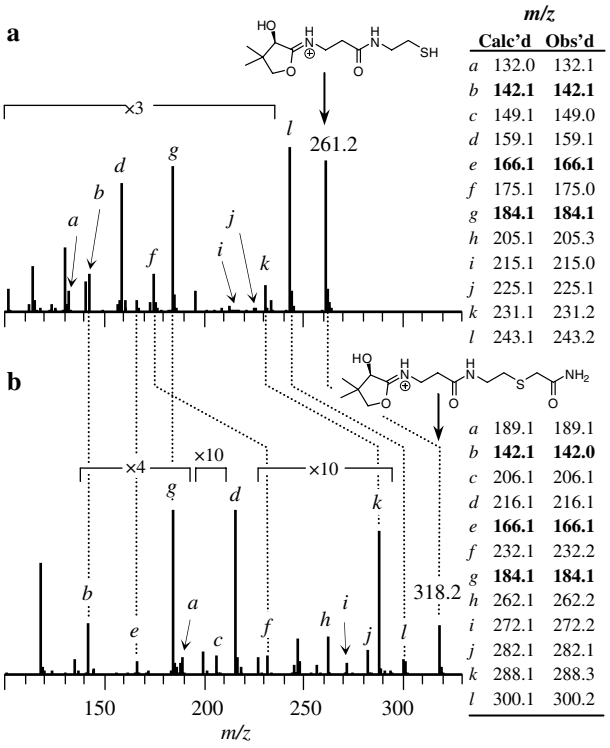

Figure 2. Confirming the diagnostic potential of the MS^3 PPant fragments. (a) The MS^3 spectrum of *holo*-CouN5, with precursors at the 16+ charge state and at the PPant fragment, includes the same 12 peaks observed for *holo*-AcpC (Fig. 1c). (b) The MS^3 spectrum of acetamide-S-CouN5, with precursors at the 15+ charge state and at the PPant fragment, shows that nine of the diagnostic peaks are shifted by the mass of the linked substrate (57 Da).

We applied our approach to confirm the phosphopantetheinylation of the acyl carrier protein AcpC after incubation with CoA in the presence or absence of Sfp. In both cases, analysis of the reaction products by nano-capillary LC-ESI and linear ion trap MS revealed charge state distributions consistent with the presence of both *holo*- and *apo*-AcpC. However, the presence of Sfp correlated with an increased proportion of *holo*-AcpC relative to *apo*-AcpC (Fig. 3a). CID on the 16+ charge state (*m/z* 796) of Sfp-treated AcpC, followed by CID with precursor at *m/z* 261, yielded peaks at *m/z* 132.0, 142.1, 149.1, 159.0, 166.1, 175.1, 184.1, 205.1, 215.2, 225.1, 231.2, and 243.2 (Fig. 3b), which agree with the pattern of MS^3 ions observed for *holo*-CouN5 (Fig. 1c).[22] These results indicate that AcpC was phosphopantetheinylated by Sfp and, to a lesser extent, by an endogenous PPTase of *E. coli*. One such protein is the 14.1-kDa protein AcpS (Accession No. P24224).[11] To determine whether AcpS can phosphopantetheinylate AcpC, we incubated the latter with a BODIPY® FL-linked CoA derivative[12] together with either Sfp or AcpS. SDS–PAGE analysis of both the Sfp and AcpS

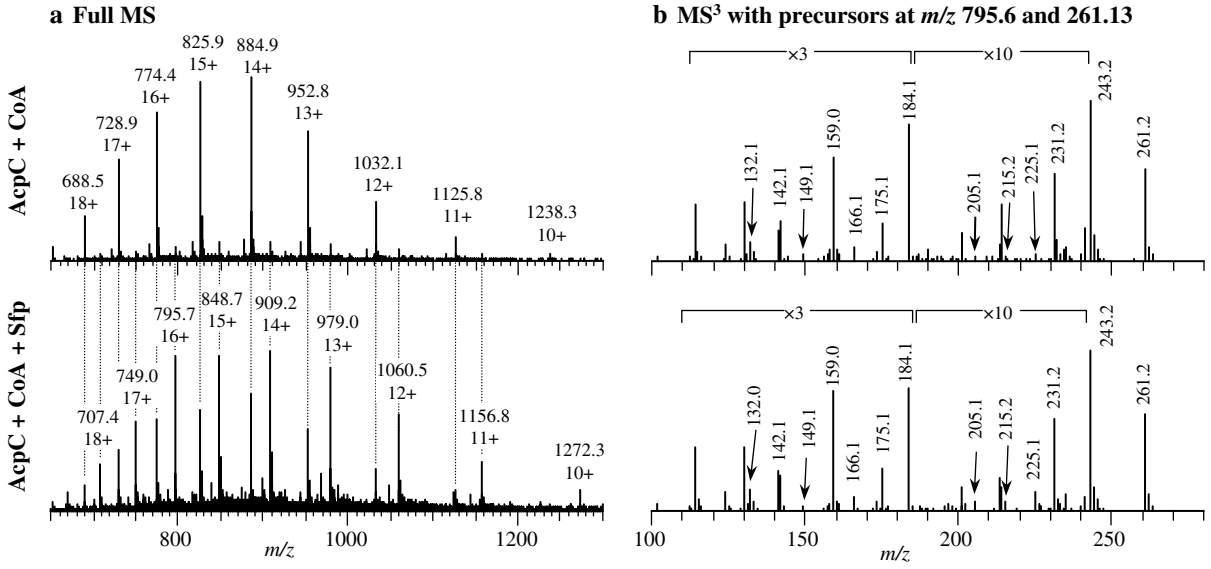

Figure 3. Sfp and AcpS can phosphopantetheinylate AcpC. (a) Broadband MS spectra of AcpC, enriched after overexpression in *E. coli* and incubated with CoA in the absence (top) or presence (bottom) of Sfp, indicate that the amount of *holo*-AcpC increased relative to *apo*-AcpC when Sfp was present. (b) The MS^3 spectra of the same samples shown in part (a), with precursors at the 16+ charge state and at the PPant fragment, show that *holo*-AcpC was present in both cases.

reaction products gave fluorescent bands consistent with the molecular weight of AcpC (11.4 kDa), indicating that both Sfp and AcpS can phosphopantetheinylate AcpC (Fig. 4).[23]

Our investigations to date show that ion trap mass spectrometers capable of MS^3 experiments can be used to recognize phosphopantetheinylated carrier proteins of PKS and NRPS pathways and to determine the masses of substrates bound to the *holo* form of such proteins. The results from our first application of the proposed method indicate that AcpC can be phosphopantetheinylated by two PPTases of rather different substrate specificity. Since expression of AcpC is required for production of hemolytic activity in GBS,[6] our results suggest that formation of *holo*-AcpC may also be necessary. We propose that this post-translational modification may be accomplished by CylK (Accession No. NP_735106), another product of the *cyl* gene cluster with very low homology to Sfp and CesP, the PPTase of *Bacillus cereus*.[13] Interestingly, in-frame deletion of *cylK* abolishes most, but not all, of the hemolytic activity of GBS.[14] Since we have shown that *E. coli* AcpS can phosphopantetheinylate AcpC, the residual hemolytic activity of the *cylK* deletion mutants may be due to phosphopantetheinylation of AcpC by a 'borrowed' PPTase, such as the 4′-phosphopantetheinyl transferase of *S. agalactiae* NEM316 (Accession No. NP_736164), a protein with 50% similarity to AcpS of *E. coli*, and therefore likely involved in fatty acid biosynthesis.

In conclusion, our top-down approach based on multiple stages of tandem MS has the potential to facilitate the study of NRPS and PKS systems with low-resolution ion trap mass spectrometers. Possible applications of this method include the routine characterization of orphan gene clusters,[15] the implementation of substrate screens, and the identification of phosphopantetheinylated proteins in a proteomic setting on LC time scales. This method may benefit other groups having access to affordable ion trap instruments but not to the costly FTICR-MS instruments currently used for top-down characterization of NRPS and PKS pathways.

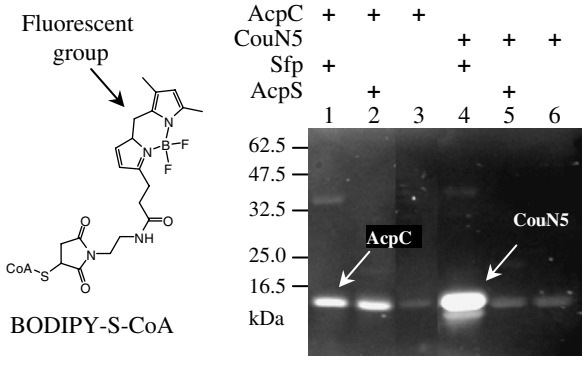

Figure 4. AcpC was fluorescently labeled by incubation with BODIPY-S-CoA (left) in the presence of Sfp (lane 1) or AcpS (lane 2). For comparison, *apo*-CouN5 was found to be labeled in the presence of Sfp (lane 4) but not in the presence of AcpS (lane 5).

Acknowledgments

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4. For example, the PPant peak resulting from an acyl carrier protein loaded with malonic acid can be predicted to be at *m/z* 345.14787 = 261.12674 (PPant fragment, $C_{11}H_{21}N_2O_3S^+$) + 102.03169 (malonic acid, $C_4H_6O_3$) − 18.01056 (water, H_2O).
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16. The *acpC* gene from hemolytic GBS strain NCTC 10/84 (serotype V) was amplified with primers 5′-atgattaactgg gctgaagaacgtaa-3′ (forward) and 5′-cttagcttggtcatctatat cctcact-3′ (reverse), based on published sequences for the GBS serotype V strain 2603 V/R,[24] and the resulting

302-bp amplicon was gel-purified and cloned into a pEXP5-CT/TOPO (Invitrogen) vector according to manufacturer's instructions, in-frame with a downstream His_6 tag, but omitting the native stop codon. Transformants were selected on ampicillin (100 μg/mL), screened by PCR using T7 and gene-specific primers to confirm orientation, and sequenced to confirm identity. Purified plasmids of confirmed clones were prepared using the QiaPrep Spin Miniprep kit (Qiagen), transformed into *E. coli* BL21(DE3) (Novagen), and stored at −80 °C in 5% glycerol. A 5-mL starter culture, grown overnight at 37 °C in Luria–Bertani medium (Difco) with ampicillin (50 mg/L), was added to 0.5 L of the same medium and shaken at ~250 rpm and 37 °C until $OD_{600} \approx 1.0$. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG, 100 mg/L) (APEX Fine Chemicals) and by shaking at ~250 rpm and 37 °C for 3 h. Cells were harvested by centrifugation at 3696*g* and stored at −20 °C. The cell pellets were thawed, resuspended in 20 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and lysed by sonication (Fisher Scientific, Model 500; five runs of fifteen 2-s pulses at 30% intensity). Nickel–nitrilotriacetic acid (Ni–NTA) Superflow resin (Qiagen) was used to purify AcpC-His_6 under native conditions according to the manufacturer's protocols. The protein was buffer-exchanged into 50 mM Tris–Cl, pH 7.5, 5% glycerol with a PD 10 column (GE-Amersham) and stored at −80 °C. The final protein concentration was ~3.0 mg/mL, as determined by Bradford assay, and the proportion of AcpC to other residual proteins was about 50:50, as determined by SDS–PAGE.

17. A 4-μL sample of enriched AcpC was desalted with C4 ZipTip® pipette tips (Millipore) following the manufacturer's protocol, diluted to 50 μL in 50:49:1 MeOH/H_2O/AcOH, infused by nano-electrospray ionization with a Biversa Nanomate (Advion Biosystems, Ithaca, NY), and analyzed by MS^2 (normalized collision energy: 13; width: 4; data type: profile; precursor: *m/z* 796) on a Finnigan LTQ-MS (Thermo-Electron Corporation, San Jose, CA) running Tune Plus software version 1.0 (Thermo). The final spectrum (Fig. 1b) was obtained by averaging 298 MS^2 scans with QualBrowser software version 1.4 SR1 (Thermo). The chemical formulas of the MS^3 fragment ions listed in Figure 1c were confirmed by locating the corresponding ions in MS^2 spectra of CoA, acquired with the Fourier transform ion cyclotron resonance (FTICR) mass analyzer portion of a Finnigan LTQ-FT MS.
18. The MS^3 scans of AcpC were collected and averaged as described for the MS^2 scans of AcpC (precursor *m/z*: 261; normalized energy: 14; width: 2; microscans: 10; data type: profile; average: 114 scans).[17]
19. The expression and purification of CouN5 have been described.[25] To obtain *holo*-CouN5, 20 μL of 60 μM purified and desalted CouN5 were incubated for 6 h at room temperature with 1 μL of 10 mM CoA (Aldrich), 1 μL of 150 μM Sfp (or 1 μL of 100 mM Tris–HCl, pH 7.5, for the control reaction), 5 μL of 10 mM tris(2-carboxyethyl)-phosphine (TCEP) (Fluka BioChemika), and 1 μL of 100 mM $MgCl_2$ (Teknova). Sfp was expressed and purified as described.[10]
20. Nano-capillary columns were prepared by drawing a 360-μm O.D., 100-μm I.D. deactivated, fused silica tubing (Agilent) with a Model P-2000 laser puller (Sutter Instruments Co.) (heat: 330, 325, 320; Vel: 45; Del: 125) and were packed at ~600 psi to a length of ~10 cm with C18 reverse-phase resin suspended in methanol. The column was equilibrated with 90% of solvent A (water, 0.1% AcOH) and loaded with 10 μL of a 1:20 dilution in water of the reaction mixture by flowing 90% of solvent A and 10% of solvent B (ACN, 0.1% AcOH) at 20 μL/min for 5 min, 15 μL/min for 3 min, and 10 μL/min for 12 min. Proteins were eluted at ~500 nL/min with a time-varying solvent mixture [break points (min, % of solvent A): (20, 90), (23, 75), (43, 10)] and directly electro-sprayed into the LTQ MS inlet (source voltage: 1.8 kV; capillary temperature: 180 °C). MS^n scans of holo-CouN5 (Fig. 2a) were acquired (normalized collision energy: 15, 16 for $n = 2, 3$; width: 10, 6 for $n = 2, 3$; data type: centroid; precursors: *m/z* 807.00, 261.17 for $n = 2, 3$) under the control of Xcalibur software version 1.4 SR1 (Thermo) and averaged (RT 32.22–32.39 min) using QualBrowser.
21. Acetamide-S-CouN5 was prepared by incubating 20 μL of 60 μM CouN5 for 6 h at room temperature with 2 μL of 5 mM acetamide-S-CoA, 1 μL of 150 μM Sfp, 5 μL of 10 mM TCEP, and 1 μL of 100 mM $MgCl_2$. Acetamide-S-CoA was prepared by reacting 1 μL of 100 mM acetamide (Aldrich) in DMSO with 2 μL of 10 mM CoA in water for ~10 min, and then neutralizing excess acetamide with 1 μL of 100 mM β-mercaptoethanol in DMSO. MS^n scans of Acetamide-S-CouN5 (Fig. 2b) were acquired (MS^n precursors: *m/z* 811.00, 318.15 for $n = 2, 3$) and averaged (RT 31.96–32.15 min) as described for *holo*-CouN5.[20]
22. *Holo*-AcpC was prepared by incubating 200 μL of 30 μM enriched AcpC with 10 μL of 10 mM CoA, 4 μL of 10 mM TCEP, 4 μL of 100 mM $MgCl_2$, and 5 μL of 150 μM Sfp for ~16 h at room temperature. Broadband MS and MS^3 scans of a 1:7 dilution of Sfp-treated AcpC in water (bottom of Fig. 3) were acquired (MS^n precursors: *m/z* 795.6, 261.13 for $n = 2, 3$) and averaged (RT 33.6–41.00 min) as described for *holo*-CouN5.[20] Scans of untreated AcpC (top of Fig. 3) were similarly acquired and averaged (RT 32.10–42.00 min).
23. Preparation of BODIPY-S-CoA from CoA and BODIPY® FL *N*-(2-aminoethyl)maleimide (Invitrogen) was described.[12] CouN5 and AcpC were separately incubated for 1 h at room temperature with catalytic amounts of Sfp, AcpS, or Tris–HCl, pH 7.6, buffer, in the presence of BODIPY-S-CoA and $MgCl_2$. The products were analyzed by 16% SDS–PAGE (PAGEgel, Inc.) and the resulting bands were visualized by fluorescence imaging with an AlphaImager® HP (Alpha Innotech).
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