Published in final edited form as:

Methods Enzymol. 2009 ; 459: 143–163. doi:10.1016/S0076-6879(09)04607-2.

Formation and Characterization of Acyl Carrier Protein-Linked Polyketide Synthase Extender Units

Yolande A. Chan and Michael G. Thomas*

Department of Bacteriology University of Wisconsin-Madison Madison, WI 53706
yachan@wisc.edu Phone: 608-265-7767 Fax: 608-262-9865

Abstract

Polyketide natural products are assembled by the condensation of an initiating precursor, or starter unit, with a series of additional precursors referred to as extender units. While there are a number of polyketide synthase starter units, there are currently only seven known polyketide synthase extender units. Polyketide synthase extender units thioesterified to coenzyme A have been known for some time; however, polyketide synthase extender units thioesterified to acyl carrier proteins (ACPs) have been identified only recently. Two of them, (2*R*)-hydroxymalonyl-ACP and (2*S*)-aminomalonyl-ACP, are found in the biosynthetic pathway of the antibiotic zwittermicin A in *Bacillus cereus* UW85. The focus of this chapter is the *in vitro* formation of (2*R*)-hydroxymalonyl-ACP and (2*S*)-aminomalonyl-ACP and the characterization of these extender units using high performance liquid chromatography and matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

Introduction

Polyketide synthases (PKSs) elongate the backbone of a polyketide using extender units, thioesterified carboxylic acid precursors covalently bound to either coenzyme A (CoA) or the 4'-phosphopantetheinyl (4'-Ppant) groups of holo-acyl carrier proteins (ACPs) (Chan et al., 2009). Those precursors bound to CoA are known as CoA-linked PKS extender units and include malonyl-CoA, (2*S*)-methylmalonyl-CoA, (2*S*)-ethylmalonyl-CoA, and the newly discovered chloroethylmalonyl-CoA. The ACP-linked PKS extender units include (2*R*)-methoxymalonyl-ACP, (2*R*)-hydroxymalonyl-ACP, and (2*S*)-aminomalonyl-ACP, which elongate a polyketide with methoxyacetyl, glycolyl, or glycyl units, respectively. The ACP-linked PKS extender units are attractive as potential tools for combinatorial biosynthesis because the substituents on the α -carbons (methoxy, hydroxyl, and amino groups) confer functionalities and hydrogen-bonding potential not available through the use of any of the currently known CoA-linked extender units. The “unnatural” natural products generated through the heterologous introduction of these extender units may have enhanced biological or therapeutic activities; furthermore, the introduction of amino or hydroxyl groups could provide chemically reactive handles that could facilitate downstream semi-synthetic chemical modifications to generate even more structural derivatives. An important first step towards the generation of new bioactive molecules using combinatorial biosynthesis is to understand how these ACP-linked PKS extender units are biosynthesized and incorporated.

The biosynthesis of ACP-linked PKS extender units is also of great interest because of their occurrence in the biosynthetic pathways of some natural products with important biological and pharmacological activities. The first ACP-linked PKS extender unit to be proposed, (2*R*)-

*(Corresponding author) Michael G. Thomas Department of Bacteriology University of Wisconsin-Madison Madison, WI 53706
thomas@bact.wisc.edu Phone: 608-263-9075 Fax: 608-262-9865.

methoxymalonyl-ACP, is a precursor to a number of methoxyacetyl-containing polyketides, including FK520 (an immunosuppressant), ansamitocin (an anticancer agent), and soraphen (an antifungal) (Ligon et al., 2002; Wu et al., 2000; Yu et al., 2002). Labeling studies and extensive genetic investigations with a number of methoxyacetyl-containing polyketides have resulted in the identification of a set of five genes encoding enzymes involved in the biosynthesis of (2*R*)-methoxymalonyl-ACP (Byrne et al., 1993; Carroll et al., 2002; Kato et al., 2002; Reeves et al., 2002; Schuhmann et al., 2007; Wu et al., 2000). In FK520 biosynthesis, the enzyme FkbH is proposed to dephosphorylate and covalently tether the glycolytic substrate 1,3-bisphosphoglycerate (1,3-bPG) to the 4'-Ppant group of an ACP, forming glyceryl-FkbJ. Next, two enzymes FkbK (NAD⁺-dependent) and FkbI (FAD-dependent) catalyze the oxidation of glyceryl-FkbJ to the intermediate (2*R*)-hydroxymalonyl-FkbJ. The methyltransferase (MT) FkbG then catalyzes the *O*-methylation of (2*R*)-hydroxymalonyl-FkbJ to form (2*R*)-methoxymalonyl-FkbJ (Fig. 1A) (Ligon et al., 2002). In soraphen biosynthesis, the proposal for (2*R*)-methoxymalonyl-ACP formation is slightly different due to the absence of an FkbH homolog (Ligon et al., 2002). SorC encodes a three-domain enzyme with an acyltransferase (AT), ACP, and MT domain. The AT domain is proposed to dephosphorylate and covalently tether 1,3-bPG to the ACP domain, forming glyceryl-SorC. This intermediate is oxidized by SorD and SorE to form (2*R*)-hydroxymalonyl-SorC, which is then methylated by the MT domain of SorC to form (2*R*)-methoxymalonyl-SorC. Alternatively, Floss and colleagues propose that the *O*-methylation of glyceryl-ACP occurs prior to the oxidation steps in the biosynthesis of soraphen and other methoxyacetyl-containing polyketides (Wenzel et al., 2006) (Fig. 1B). Their proposal that methylation precedes oxidation is based on the occurrence of the MT domain adjacent to the ACP domain of SorC. Additional work is needed to determine the order of these events. To date, (2*R*)-methoxymalonyl-ACP has not been reconstituted *in vitro*, although it has been generated *in vivo* in a heterologous system (Rude and Khosla, 2006).

While (2*R*)-methoxymalonyl-ACP is a precursor in the biosynthesis of number of polyketides, the occurrences of (2*R*)-hydroxymalonyl-ACP and (2*S*)-aminomalonyl-ACP have been more limited. These precursors occur in the biosynthesis of the antibiotic zwittermicin A and may occur in the biosynthesis of some other polyketides (Chan et al., 2009; Walton et al., 2006; Wu et al., 2000). For example, based on labeling studies, (2*R*)-hydroxymalonyl-ACP is a likely precursor in the biosynthesis of aflastatin A, an inhibitor of aflatoxin production (Ono et al., 1998). Future studies may also reveal its occurrence in the biosynthesis of other polyketides, such as amicoumacin B (Gebhardt et al., 2002) and galantin (Shoji et al., 1975).

(2*R*)-hydroxymalonyl-ACP and (2*S*)-aminomalonyl-ACP have been reconstituted *in vitro* using heterologously overproduced zwittermicin A enzymes (Chan et al., 2006). We have shown that (2*R*)-hydroxymalonyl-ACP formation proceeds in the following way: the substrate 1,3-bPG is dephosphorylated and covalently tethered to the 4'-Ppant group of holo-ZmaD, forming glyceryl-ZmaD, which is subsequently oxidized by ZmaG and ZmaE to form the final product, (2*R*)-hydroxymalonyl-ZmaD (Fig. 1C). In our studies of (2*S*)-aminomalonyl-ACP formation, we have shown that the adenylation (A) domain, ZmaJ, activates and covalently tethers L-serine to the 4'-Ppant group of holo-ZmaH to form seryl-ZmaH. ZmaG and ZmaI then oxidize seryl-ZmaH to form (2*S*)-aminomalonyl-ZmaH (Fig. 1D).

In this chapter we describe the *in vitro* reconstitution of (2*R*)-hydroxymalonyl-ACP and (2*S*)-aminomalonyl-ACP using heterologously purified enzymes from zwittermicin A biosynthesis and the characterization of these precursors using high performance liquid chromatography (HPLC) and matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS).

Overproduction and Purification of Recombinant Proteins

Principle

Heterologous overproduction in *Escherichia coli* of the zwittermicin A biosynthetic enzymes yields soluble and functional proteins, facilitating the *in vitro* reconstitution of (2*R*)-hydroxymalonyl-ZmaD and (2*S*)-aminomalonyl-ZmaH. The use of constructs with N-terminal hexa-histidine tags allows the biosynthetic enzymes to be purified readily in high yield and with high purity using affinity chromatography with a nickel-based resin. The hexa-histidine tag of the protein-of-interest binds to the nickel resin, and the protein is eluted with increasing concentrations of imidazole, which has a higher affinity for the nickel resin. Here we describe the procedures for overproducing and purifying the enzymes required for forming (2*R*)-hydroxymalonyl-ZmaD and (2*S*)-aminomalonyl-ZmaH: ZmaD, ZmaE, ZmaG, ZmaN, ZmaH, ZmaI, and ZmaJ.

Materials

Competent cells of *E. coli* strain BL21 (λ DE3) in 100 μ L aliquots, stored at -80°C

Plasmid preparations of overexpression constructs for each of the biosynthetic genes: pET28b-*zmaD*, -*zmaE*, -*zmaG*, -*zmaH*, -*zmaI*, -*zmaJ*, and -*zmaN* (kanamycin-resistant)

Plasmid preparations of an overexpression construct for *Bacillus subtilis* phosphopantetheinyl transferase: pET29-*sfp* (kanamycin-resistant) (Quadri et al., 1998)

Kanamycin: 50 mg/mL, filter-sterilized with 0.2 μ m filter

Lysogeny broth (LB) medium: Autoclave a mixture containing 10 g tryptone, 5 g yeast extract, and 10 g NaCl per 1 L distilled H_2O (dH_2O).

Lysogeny broth (LB) kanamycin agar: Prepare as described for LB medium, but add 15 g agar per 1 L dH_2O . After autoclaving, cool to 65°C and add kanamycin to 50 $\mu\text{g}/\text{mL}$. Pour 25 mL per plate.

Isopropyl- β -D-thiogalactopyranoside (IPTG): 0.1 M filter-sterilized with 0.2 μ m filter Tris (hydroxymethyl)aminomethane (Tris)

NaCl

Glycerol

His-tag purification buffer, prepared freshly: Using double-distilled H_2O (ddH_2O), prepare 300 mL of 20 mM Tris, 300 mM NaCl, 10% (v/v) glycerol, titrated to pH 8.0 with concentrated HCl.

Nickel-nitroloacetic acid (Ni-NTA) agarose resin, a 1:1 slurry of resin and 20% ethanol (Qiagen, Valencia, CA)

Imidazole: Using ddH_2O , prepare 250 mL of a 2 M solution and filter-sterilize using a Nalgene (Rochester, NY) filtration bottle. Protect solution from light by covering bottle in foil. Store at 4°C .

2 $\times$ Cracking buffer: Prepare 10 mL by adding the following: 1.2 mL Tris pH 6.8, 100 μL β -mercaptoethanol, 500 μL of 20% Sodium dodecyl sulfate (SDS) solution, 2.5 mL of 50% glycerol solution, and 250 μL of 0.4% bromothymol blue solution.

Tris-Cl polyacrylamide gel (12% resolving gel, 5% stacking gel) with ten 30 μL wells

5 $\times$ SDS-polyacrylamide gel electrophoresis (PAGE) running buffer: Dissolve 72 g glycine, 15 g Tris base, 5 g SDS in 1 L dH_2O , pH 8.3.

1× SDS-PAGE running buffer: Using dH₂O, mix 200 mL of 5× SDS-PAGE running buffer in a final volume of 1 L.

Broad-range protein molecular weight standard (Bio-Rad, Hercules, CA)

0.1% (w/v) Coomassie R-250 stain solution: Prepare by dissolving 1 g Coomassie R-250 in a 1 L solution containing 50% methanol and 10% acetic acid.

Destain solution: Prepare 1 L solution containing 45% methanol and 10% acetic acid.

Dialysis buffer for all proteins except ZmaG, prepared freshly: Using ddH₂O, prepare 2 × 1 L (for each protein) consisting of 50 mM Tris, 100 mM NaCl, 10% (v/v) glycerol, titrated to pH 8.0 with concentrated HCl.

Dialysis buffer for ZmaG, prepared freshly: Prepare the same way as above dialysis buffer, but substitute 10% sucrose (w/v) for glycerol.

Snakeskin® Pleated Dialysis Tubing (Pierce, Rockford, IL): 3,000-Da molecular weight cut-off (MWCO) and 10,000-Da MWCO

Dialysis clips and foam float

Millipore Centriprep® protein concentrators: YM-10 and YM-30 (10,000- and 30,000-Da MWCO)

Liquid nitrogen: Obtain ~200 mL just before use.

Heterologous Overproduction of Proteins

Procedure—For optimal overproduction of the histidine-tagged proteins, each expression construct is freshly introduced by transformation into competent *E. coli* BL21 (λDE3) for heterologous overexpression. A microcentrifuge tube containing 100 μL of competent *E. coli* BL21 (λDE3) is thawed on ice and transferred to a sterile pre-chilled glass tube. Plasmid DNA (50-250 ng) is added to the competent cells, and the tube is incubated on ice. After 30 min, the cells are heat-shocked in a 42°C water bath for 60 seconds, iced for 60 seconds, and 800 μL of fresh LB medium is added to the tube. The cells are allowed to recover for 50 min, growing in a 37°C incubator shaker. Following recovery, 50-100 μL of the cells is plated to LB kanamycin agar, and the plate is incubated at 37°C for ~16-18 h. An average-sized colony is used to inoculate 50 mL of LB kanamycin (50 μg/mL) broth in a shake flask, which is incubated ~16-18 h on a 37°C shaker. LB kanamycin (50 μg/mL) broth (3 × 1L, in 2.8 L shake flasks) is inoculated with 10 mL of the overnight culture and grown at 25°C and 180 rpm. When OD₆₀₀ reaches 0.4-0.6, the temperature is dropped to 15°C for 2 h, after which the cells are induced by the addition of IPTG at a final concentration of 60 μM. The cells are grown an additional 16-18 h at 15°C, 180 rpm. Cells are harvested by centrifugation at 7,000 rpm (Beckman Model J2-21 centrifuge, KA-9 KompSpin® rotor) at 4°C, for 10 min. The cell pellets are transferred to tared 50 mL polypropylene tubes, and the wet weight of the cells is determined. Cell pellets can be used immediately or can be stored at -20°C for 1-2 weeks, if necessary. If they are used at a later date, the frozen cells should be thawed on ice prior to proceeding with the purification.

Purification of Enzymes Using Batch-Binding Method with Nickel-NTA Resin

Procedure—All materials involved in the purification steps, including tubes, buffers, cells, cell extracts, and Ni-NTA resin, should be kept at 4°C or on ice. The 5× and 1× SDS-PAGE running buffers, 0.1% Coomassie-blue stain solution, and Destain solution may be kept at 22°C.

Cell pellets are resuspended in a volume (in mL) of his-tag purification buffer that is three times the wet weight (in g). Cells should be resuspended gently, without vortexing. Cells are lysed with two passages through a French pressure cell set at 1200 psi. Alternatively, cells may be lysed by sonication (Fisher 550 Sonic Dismembrator, power = 5, 15 min sonication with 1 s on, 1 s off). The lysed cells are centrifuged at 15,000 rpm (Beckman Model J221 centrifuge, JA-25.5 rotor), 4°C, for 30 min. During centrifugation, the Ni-NTA agarose resin is prepared. One mL of the resuspended resin slurry is pipetted into each of two 50-mL polypropylene tubes. The 20% ethanol in which the resin is stored is washed away by adding 40 mL cold ddH₂O to each of the resin-containing tubes and centrifuging at 2,000 rpm (Beckman Allegra™ 6R centrifuge), 4°C, for 5 min. The supernatant is carefully discarded by decanting or pipetting, and 40 mL cold his-tag buffer is added to the resin before centrifuging at 2,000 rpm (Beckman Allegra™ 6R centrifuge), 4°C, for 5 min. The resin should not be allowed to dry out at any time. Following centrifugation, the tubes containing resin with buffer are placed on ice. When the centrifugation of the cell-free extract is complete, the clarified cell-free extract is carefully decanted or pipetted into pre-chilled polypropylene tubes, and imidazole is added to a final concentration of 5 mM. The his-tag buffer in the resin-containing tubes can then be removed and discarded, and the cell-free extract is added to the resin. The lids of the tubes are securely closed, and the tubes are placed flat in an ice bucket, which is then put on a flat-top shaker set at the lowest setting to gently agitate for 1-2 h. Next, the tubes are centrifuged at 2,000 rpm (Beckman Allegra™ 6R centrifuge), 4°C, for 5 min to pellet the resin, and the supernatant is carefully pipetted off. Immediately, using a small volume of his-tag buffer (5 mM imidazole) and a glass Pasteur pipet, the resin is gently transferred to a 25-mL column equipped with a stopcock. During the transfer, the stopcock should be closed. After the beads have settled, the stopcock is opened to drain the buffer until a small amount remains above the resin bed. The resin is then washed with 10 mL of his-tag buffer containing 5 mM imidazole, and the protein is eluted with a stepwise gradient of 5 mL of his-tag buffer containing increasing concentrations of imidazole (20, 40, 60, 100, and 250 mM). Each fraction is collected into clean glass tubes on ice. ZmaE and ZmaI, the acyl-ACP dehydrogenases, purify with their FAD cofactors, resulting in vibrant yellow fractions.

To determine which elutions contain purified protein, SDS-PAGE followed by Coomassie-blue staining of the gel is performed. Aliquots (10 µL) from each fraction are added to microcentrifuge tubes containing 10 µL of 2× cracking buffer. The samples are boiled at 100°C for 5 min, cooled to 22°C, and centrifuged briefly to remove the condensation from the lids of the tubes. A Tris-Glycine polyacrylamide gel is run with 10 µL of each sample and 5 µL of the protein molecular weight standard. The gel is run at 150 V for 1 h, placed in a glass dish with a tight-fitting lid, stained with 0.1% Coomassie R-250 stain solution for 15 min, rinsed in ddH₂O, and destained in 50 mL of destain solution. The destain solution can be changed to fresh solution to continue destaining, if necessary. Based on observations from the gel, fractions containing purified protein are pooled and dialyzed in Snakeskin® pleated dialysis tubing. All enzymes are dialyzed in 10,000-Da MWCO dialysis tubing, except for ZmaD and ZmaH, which should be dialyzed in 3,000-Da MWCO tubing. To set up the dialysis, the tubing is cut to an appropriate size to contain the pooled fractions, pre-wet with the appropriate dialysis buffer, and closed at one end with the use of a dialysis clip. The protein is then carefully transferred to the tubing, and the second dialysis clip is fastened securely to seal the tubing. The foam float is affixed to one of the clips, and the dialysis assembly is placed in the dialysis buffer. It should be noted that the dialysis buffer for ZmaG should contain sucrose instead of glycerol, to minimize any potential inhibition of enzymatic activity by glycerol. With gentle stirring, dialysis should occur at 4°C for 4-16 h, followed by an additional 4 h in fresh dialysis buffer. During dialysis, ZmaE and ZmaI retain their cofactors and remain yellow. Following dialysis, the protein is concentrated using Millipore Centriprep® YM-10 or YM-30 protein concentrators, following the manufacturer's instructions. Proteins are concentrated to about 1 mg/mL, and protein concentrations are determined from absorbances at 280 nm and the

calculated molar extinction coefficients (ZmaD, 2,560 M⁻¹cm⁻¹; ZmaE, 44,410 M⁻¹cm⁻¹; ZmaG, 21,180 M⁻¹cm⁻¹; ZmaH, 2,560 M⁻¹cm⁻¹; ZmaI, 44,770 M⁻¹cm⁻¹; ZmaJ, 46,760 M⁻¹cm⁻¹; and ZmaN, 41,070 M⁻¹cm⁻¹). As soon as possible, the protein should be flash-frozen by pipetting the protein, drop by drop, into a Dewar containing about 200 mL of liquid nitrogen. The frozen protein pellets can then be transferred to cryotubes that have been cooled in dry ice. Protein is stored stably at -80°C until further use. Per L of culture, this procedure results in approximately 2-6 mg of ZmaE, ZmaG, ZmaI, ZmaJ, and ZmaN and approximately 6-8 mg of ZmaD and ZmaH (Fig. 2). We note that the same purification procedure can be followed to purify histidine-tagged Sfp.

Formation and Characterization of Hydroxymalonyl-ACP and Aminomalonyl-ACP

In Vitro Phosphopantetheinylation of the ACPs ZmaD and ZmaH

Principle—As purified from *E. coli* as described above, the majority of ZmaD and ZmaH is in the inactive apo-form. Enzymes responsible for catalyzing the conversion from the apo- to holo-forms are known as phosphopantetheinyl transferases (PPTases), which transfer the 4'-Ppant group from CoA to a conserved serine residue of an ACP (Lambalot et al., 1996). One such PPTase from *B. subtilis* is Sfp, which has been shown to act on a wide range of ACPs (Quadri et al., 1998). While phosphopantetheinylation is sometimes performed *in vivo* by co-expressing an ACP-overexpression construct and a PPTase-overexpression construct (Gokhale et al., 1999), we have found that this method has resulted in a significant proportion of inactive ACPs, presumably due to failed and/or improper phosphopantetheinylation. We have found that performing *in vitro* phosphopantetheinylation by incubating purified Sfp with the ACP of interest results in nearly complete conversion from the apo- to the holo-form, as determined by the HPLC-based procedure described later. The conversion of apo- to holo-ACPs using *E. coli* ACP synthase has been described previously; here we present a modified protocol for preparing holo-ZmaD and holo-ZmaH (Lambalot and Walsh, 1997).

Materials—

Tris buffer: 0.75 M, pH 7.5

MgCl₂: 0.2 M

Tris(2-carboxyethylphosphine) TCEP: 50 mM, pH 7.5

Coenzyme A (CoA): 20 mM and 2 mM

Apo-ZmaD, Apo-ZmaH, and Sfp: As purified in the above procedure.

Procedure—In a final volume of 200 µL, the reaction mixture for making holo-ZmaD contains 75 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM TCEP, 500 µM CoA, 12.5 µM ZmaD, and 1 µM Sfp. The reaction mixture for making holo-ZmaH contains 75 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM TCEP, 50 µM CoA, 12.5 µM ZmaH, and 1 µM Sfp. The reactions are carried out in 1.5 mL microcentrifuge tubes and incubated at 22°C for 1 h to ensure maximal conversion. It should be noted that the apo- to holo- conversion for ZmaD proceeds less readily than that for ZmaH; thus, the reaction with ZmaD requires a higher concentration of CoA. A high amount of the ACP relative to Sfp (or other catalytic enzymes) is necessary because the ACP does not function catalytically in the reactions; rather, the ACP is the substrate and the modified ACP is the product, and sufficient quantities of ACP are required for detection.

HPLC-based Characterization of Modified ACPs

Principle—Reverse-phase HPLC with a C18 peptide column is used to monitor the elution of peptides and proteins, based on their absorbance at 220 nm. We describe a method to detect the modifications of an ACP (i.e. apo- to holo- conversion) based on the different elution times of the ACP species from the HPLC column.

Materials—

ACP-containing reaction mixtures to analyze (i.e. apo- and holo-ACP reactions)

Analytical C18 peptide (mass spec) HPLC column, 4.6 × 250 mm, 5 µm particle size, 300 Å, (catalog #218MS54, Vydac) and associated guard column (catalog #218GD54, Vydac)

HPLC Solvent A: Filter 1 L ddH₂O with 0.5 µm filter. Add trifluoroacetic acid (TFA) to a final concentration of 0.1% (vol/vol).

HPLC Solvent B: Prepare 500 mL of HPLC-grade acetonitrile (ACN), 0.1% TFA (vol/vol)

CO₂(s)/ethanol bath: Prepare by adding 95% ethanol to crushed CO₂(s) in a styrofoam container

Procedure—Samples to be analyzed are prepared. These may include samples prepared as described above for the *in vitro* phosphopantetheinylation of ZmaD and ZmaH; they may also include samples prepared according to the procedures described below for forming (2*R*)-hydroxymalonyl-ZmaD and (2*S*)-aminomalonyl-ZmaH. Samples should contain relatively low glycerol concentrations, as glycerol can negatively affect peak resolution. Therefore, the enzyme preparations (in glycerol-containing buffers) should be at sufficiently high concentrations to minimize the amount of glycerol added to the reactions by the addition of enzymes.

A sample (100–260 µL) is injected onto the column, which has been equilibrated first with a mixture of 80% solvent A/ 20% Solvent B at a flow rate of 1 mL/min. The following program is used, with the flow rate set at 1 mL/min: 5 min at 80% A/ 20% B; 20 min linear gradient from 80% A/ 20% B to 20% A/ 80% B; 10 min isocratic at 20% A/ 80% B; 1 min linear gradient from 20% A/ 80% B to 80% A/ 20% B; 10 min isocratic at 80% A/ 20% B.

The absorbance at 220 nm is monitored, with peaks corresponding to the elution of proteins. The peak corresponding to the ACP of interest may be collected in colorless microcentrifuge tubes (to minimize the potential for “extractables” by ACN), flash frozen in a CO₂(s)/ethanol bath, and stored at –80°C for further analysis (see below for MALDI-TOF MS analysis procedure).

Figure 3 shows HPLC traces for apo- and holo-ZmaD, as well as apo- and holo-ZmaH. The addition of 4'-Ppant prosthetic groups to the apo-ACPs results in shorter elution times for both holo-ACPs. The percent conversion from the apo- to holo- forms can be determined by calculating the area under the peaks. This method also enables one to determine how much holo-ACP has converted to an acylated or other form.

Formation of (2*R*)-Hydroxymalonyl-ACP

Principle—(2*R*)-Hydroxymalonyl-ZmaD formation is reconstituted *in vitro*, using 1,3-bPG and the enzymes ZmaD, ZmaN, ZmaG, and ZmaE (Fig. 1C). The omission of ZmaG and ZmaE from the reaction mixture results in the formation of glyceryl-ZmaD, a precursor to (2*R*)-hydroxymalonyl-ZmaD. The glycolytic substrate 1,3-bPG is not commercially available and therefore must be prepared enzymatically using available materials.

Materials—

Tris buffer: 0.75 M, pH 7.5

MgCl₂: 0.2 M

TCEP: 50 mM, pH 7.5

Coenzyme A (CoA): 20 mM

(Apo-)ZmaD, Sfp, ZmaN, ZmaG, ZmaE: As purified in the above procedure.

NAD⁺: 13 mM

FAD: 6.5 mM

3-Phosphoglycerate phosphokinase (3-PGPK): 0.5 U/μL

D(-)3-Phosphoglycerate (3-PG): 10 mM

Adenosine triphosphate (ATP): Freshly prepare 0.2 M solution. Titrate to pH 7 with 5 N NaOH.

Potassium phosphate: 0.1 M, pH 7.5

DL-Glyceraldehyde-3-phosphate (G3P): 25 mM

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 0.5 U/μL

Procedure—The glycolytic substrate 1,3-bPG can be prepared using a couple of different methods (Fig 4). The first method involves the ATP-dependent phosphorylation of 3-PG by 3-PGPK to form 1,3-bPG. The second method involves the NAD⁺-dependent oxidation and phosphorylation of G3P to form 1,3-bPG. The enzymes and the substrates required for these conversions are available through Sigma-Aldrich (St. Louis, MO).

To make (2*R*)-hydroxymalonyl-ZmaD using 3-PG and 3-PGPK as a source of 1,3-bPG, the following reaction is prepared in a final volume of 260 μL: 75 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM TCEP, 500 μM CoA, 12.5 μM ZmaD, 1 μM Sfp, 1 μM ZmaN, 200 μM NAD⁺, 100 μM FAD, 1 μM ZmaG, 1 μM ZmaE, 1 U 3-PGPK, 250 μM 3-PG, and 5 mM ATP. Prior to adding ATP, the reaction is incubated at 22°C for 1 h to allow for the conversion of apo- to holo-ZmaD. The complete reaction is initiated by the addition of ATP and incubated at 22°C for 40 min. It is also possible to form (2*R*)-hydroxymalonyl-ZmaD in a slightly different way, by pre-forming glyceryl-ZmaD prior to the addition of ZmaG and ZmaE. In this method, a reaction to form holo-ZmaD is set up and incubated at 22°C for 1 h. This reaction is in a volume of 200 μL and contains 75 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM TCEP, 500 μM CoA, 12.5 μM ZmaD, and 1 μM Sfp. Next, only the components involved in acylating holo-ZmaD are added (ZmaN, 3-PGPK, and 3-PG), and the reaction is incubated at 22°C for 30 min. A sample of the reaction can be saved for at this point for analysis by HPLC and MALDI-TOF MS to ensure the intermediate glyceryl-ZmaD has been formed. Finally, the rest of the components are added to form (2*R*)-hydroxymalonyl-ZmaD (200 μM NAD⁺, 100 μM FAD, 1 μM ZmaG, and 1 μM ZmaE; final volume of 260 μL), and the reaction is incubated at 22°C for 40 min.

Our group and others have observed that commercially available 3-PG is contaminated with a trace amount of 3-PGPK (Chan et al., 2006; Dorrestein et al., 2006). A reaction containing all the above components except for added 3-PGPK results in the formation of (2*R*)-hydroxymalonyl-ZmaD, although this occurs at a much slower rate than when 3-PGPK is added (1.44 nmol/min with 3-PGPK, 0.08 nmol/min without 3-PGPK) (Chan et al., 2006). The second method for making 1,3-bPG bypasses this issue by utilizing commercially available G3P and

GAPDH. Using this method, the following reaction mixture is set up in a final volume of 150 μ L: 75 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM TCEP, 500 μ M CoA, 12.5 μ M ZmaD, 1 μ M Sfp, 1 μ M ZmaN, 200 μ M NAD^+ , 100 μ M FAD, 1 μ M ZmaG, 1 μ M ZmaE, 250 μ M G3P, and 1 U GAPDH. Holo-ZmaD is pre-formed by adding the following components and incubating the mixture at 22°C for 1 h: Tris pH 7.5, $MgCl_2$, TCEP, CoA, ZmaD, and Sfp. The rest of the components are then added to the tube, and the reaction is incubated at 22°C for 40 min. It is also possible to pre-form glyceryl-ZmaD prior to forming (2*R*)-hydroxymalonyl-ZmaD by omitting ZmaG, ZmaE, and their cofactors.

HPLC analysis of reactions containing glyceryl-ZmaD and (2*R*)-hydroxymalonyl-ZmaD is performed, as described in the preceding section. As shown in Fig. 5, there is a shift in the elution time of glyceryl-ZmaD relative to holo-ZmaD. For the complete reaction, there is a slight shift in the elution time relative to the glyceryl-ZmaD reaction. As discussed in a later section, these samples are analyzed further by MALDI-TOF MS.

Formation of (2*S*)-Aminomalonyl-ACP

Principle—(2*S*)-Aminomalonyl-ZmaH formation can be reconstituted *in vitro*, using L-serine and the enzymes ZmaH, ZmaJ, ZmaG, and ZmaI (Fig. 1D). The omission of ZmaG and ZmaI from the reaction mixture results in the formation of seryl-ZmaH, a precursor to (2*S*)-aminomalonyl-ZmaH.

Materials—

Tris buffer: 0.75 M, pH 7.5

$MgCl_2$: 0.2 M

TCEP: 50 mM, pH 7.5

Coenzyme A (CoA): 2 mM

(Apo-)ZmaH, ZmaG, ZmaI, ZmaJ, and Sfp: As purified in the above procedure.

NAD^+ : 13 mM

FAD: 6.5 mM

L-serine: 10 mM

ATP: Freshly prepare 0.2 M solution. Titrate to pH 7 with 5 N NaOH.

Procedure—To make (2*S*)-aminomalonyl-ZmaH, the following reaction is prepared in a final volume of 260 μ L: 75 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM TCEP, 50 μ M CoA, 12.5 μ M ZmaH, 1 μ M Sfp, 1 μ M ZmaJ, 200 μ M NAD^+ , 100 μ M FAD, 1 μ M ZmaG, 1 μ M ZmaI, 250 μ M L-serine, and 5 mM ATP. To pre-form holo-ZmaH, all the components except ATP are added, and the reaction is incubated at 22°C for 1 h. The complete reaction is initiated by the addition of ATP and incubated at 22°C for 40 min. It is also possible to form (2*S*)-aminomalonyl-ZmaH by pre-forming seryl-ZmaH prior to the addition of ZmaG, ZmaI, and their cofactors. In this method, a reaction to form holo-ZmaH is set up and incubated at 22°C for 1 h. This reaction is in a volume of 200 μ L and contains 75 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM TCEP, 50 μ M CoA, 12.5 μ M ZmaH, and 1 μ M Sfp. Next, ZmaJ, serine, and ATP are added to the tube, and the reaction is incubated at 22°C for 30 min. A sample of the reaction mixture can be saved at this point for analysis of seryl-ZmaH formation by HPLC and MALDI-TOF MS. Finally, the rest of the components are added (200 μ M NAD^+ , 100 μ M FAD, 1 μ M ZmaG, and 1 μ M ZmaI; final volume of 260 μ L), and the complete reaction is incubated at 22°C for 40 min.

One should note that ZmaG catalyzes the oxidation of both glyceryl-ZmaD and seryl-ZmaH and is required for the formation of both (2*R*)-hydroxymalonyl-ZmaD and (2*S*)-aminomalonyl-ZmaH. Interestingly, the enzymes catalyzing the second oxidations, ZmaE and ZmaI, are pathway-specific. Although ZmaI can function in both pathways, ZmaE can function only in the (2*R*)-hydroxymalonyl-ZmaD pathway. Likewise, ZmaN and ZmaJ are also pathway-specific, as neither enzyme can acylate/aminoacylate the non-cognate ACP.

HPLC analysis of the reactions containing seryl-ZmaH and (2*S*)-aminomalonyl-ZmaH is performed. As shown in Fig. 6, the reaction for seryl-ZmaH results in a shift in elution time, relative to holo-ZmaH. When the complete reaction is analyzed by HPLC, no shift in elution time occurs; instead, a broadening of the ZmaH-associated peak is observed. The peaks corresponding to ZmaH are collected and analyzed by MALDI-TOF MS.

MALDI-TOF MS Analysis of ACPs

Principle—MALDI-TOF MS analysis of ACPs allows one to determine whether an ACP has been modified, based on a comparison of the experimental masses with the theoretical masses for the different ACP species.

Materials—

HPLC-purified ACPs to analyze (keep frozen at -80°C)

Proteo-MassTM Protein MALDI-MS Calibration Kit (Sigma-Aldrich): includes ACN, 0.1% TFA, sinapinic acid, protein standards

50% ACN/ 0.05% TFA: Prepare 600 μL in a colorless microcentrifuge tube.

Sinapinic acid matrix: Prepare 10 mg/mL solution in 600 μL 50% ACN/ 0.05% TFA solution.

Vortex vigorously for 1 min. Let mixture settle for 10 min at bench. Matrix preparation is stable for 1 week at 22°C if stored in the dark.

Sample plate for MALDI-TOF MS (Applied Biosystems)

Procedure—Prior to MALDI-TOF MS analysis, the HPLC-purified ACP samples must be prepared by lyophilizing. The ACP-containing microcentrifuge tubes are removed from -80°C , opened, and placed inside 50 mL polypropylene tubes (1-2 samples per tube). A Kimwipe[®] is placed over the top of the polypropylene tube and secured with a rubber band. The tubes are then placed inside a lyophilizing vessel and lyophilized until the ACN has evaporated, leaving a white, powdery residue (~8 h). The lyophilized protein is resuspended in 6-20 μL ddH₂O and placed on ice until further use.

The manufacturer's instructions for preparing the protein standards apomyoglobin, cytochrome C, and bovine insulin are followed. It is advisable to aliquot the standards into several tubes to minimize the number of freeze-thaw cycles. A mixture containing the three standards is spotted onto the sample plate (spot 1 μL and 2 μL).

The samples for analysis are prepared by adding 2 μL of a sample to a colorless microcentrifuge tube containing 2 μL of sinapinic acid. Matrix and sample are mixed by pipetting, and 1-2 μL of each sample is spotted onto the sample plate. The plate is allowed to dry completely before inserting the plate into the instrument chamber. The manufacturer's instructions are followed for using the mass spectrometer, with the instrument set to linear, positive-ion mode. The standards are used to calibrate the instrument.

Table I shows the theoretical and experimental masses for the various ZmaD and ZmaH species. The masses for the complete reactions for both the (2*R*)-hydroxymalonyl-ZmaD and (2*S*)-aminomalonyl-ZmaH pathways are consistent with the decarboxylated products, glycolyl-ZmaD and glycyl-ZmaH, respectively. These decarboxylations may occur due to the instability of (2*R*)-hydroxymalonyl-ZmaD and (2*S*)-aminomalonyl-ZmaH under the assay conditions (Chan et al., 2006); nevertheless, masses consistent with glycolyl-ZmaD and glycyl-ZmaH are indicative of (2*R*)-hydroxymalonyl-ZmaD and (2*S*)-aminomalonyl-ZmaH formation.

Other Methods for Characterizing Enzymes Involved in (2*S*)-Aminomalonyl-ACP Formation

ZmaJ, the seryl-activating adenylation domain involved in (2*S*)-aminomalonyl-ZmaH formation, can be characterized using standard ATP/pyrophosphate (PP_i)-exchange assays (Santi et al., 1974). These assays allow one to detect the exchange of ³²P-isotope from [³²P]-PP_i into ATP, which only occurs through the formation of an aminoacyl-enzyme intermediate.

Thus, the formation of β γ -³²P-ATP in the presence of L-serine indicates that ZmaJ can activate the amino acid.

Other methods for characterizing ZmaH involve the use of [¹⁴C]-L-serine. To show that ZmaH is covalently modified with L-serine, standard trichloroacetic acid (TCA) precipitation assays can be performed (Gehring et al., 1997; Quadri et al., 1999). ZmaH is incubated in a reaction buffer with ZmaJ, [¹⁴C]-L-serine, and ATP; then, the proteins are precipitated with TCA and analyzed by scintillation counting. The covalent modification of ZmaH also can be detected with or without TCA precipitation using SDS-PAGE followed by phosphorimaging of the dried gel.

Other Methods for Characterizing Enzymes Involved in (2*R*)-Hydroxymalonyl-ACP Formation

With a source of radiolabeled 1,3-bPG, the acylation of ZmaD during (2*R*)-hydroxymalonyl-ZmaD formation can be characterized using TCA precipitation assays and SDS-PAGE followed by phosphorimaging. Although [¹⁴C]-1,3-bPG is not available commercially, it can be synthesized from commercially available radiolabeled precursors and enzymes. In the simplest route, [¹⁴C(U)]-G3P can be purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO) and converted to [¹⁴C]-1,3-bPG using GAPDH, as described above for the formation of (2*R*)-hydroxymalonyl-ZmaD. Alternatively, [¹⁴C]-1,3-bPG can be made using [¹⁴C(U)]-D-fructose 1,6-bisphosphate, fructose 1,6-bisphosphate aldolase, GAPDH, and triose phosphate isomerase, all of which are available through Sigma-Aldrich. The aldolase converts [¹⁴C(U)]-D-fructose 1,6-bisphosphate to [¹⁴C]-G3P and [¹⁴C]-dihydroxyacetone phosphate, the latter of which can be converted to [¹⁴C]-G3P using triose phosphate isomerase; GAPDH converts [¹⁴C]-G3P to [¹⁴C]-1,3-bPG.

References

- Byrne KM, Shaflee A, Nielsen J, Arison B, Monaghan RL, Kaplan L. The biosynthesis and enzymology of an immunosuppressant, immunomycin, produced by *Streptomyces hygroscopicus* var, *ascomyceticus*. Dev. Ind. Microbiol 1993;32:29–45.
- Carroll BJ, Moss SJ, Bai L, Kato Y, Toelzer S, Yu TW, Floss HG. Identification of a set of genes involved in the formation of the substrate for the incorporation of the unusual “glycolate” chain extension unit in ansamitocin biosynthesis. J. Am. Chem. Soc 2002;124:4176–4177. [PubMed: 11960423]
- Chan YA, Boyne MT II, Podevels AM, Klimowicz AK, Handelsman J, Kelleher NL, Thomas MG. Hydroxymalonyl-acyl carrier protein (ACP) and aminomalonyl-ACP are two additional type I polyketide synthase extender units. Proc. Natl. Acad. Sci USA 2006;103:14349–14354. [PubMed: 16983083]
- Chan YA, Podevels AM, Kevany BM, Thomas MG. Biosynthesis of polyketide synthase extender units. Nat. Prod. Rep. 2009DOI: 10.1039/b801658p

- Dorrestein PC, Van Lanen SG, Li W, Zhao C, Deng Z, Shen B, Kelleher NL. The bifunctional glyceryl transferase/phosphatase OzmB belonging to the HAD superfamily that diverts 1,3-bisphosphoglycerate into polyketide biosynthesis. *J. Am. Chem. Soc* 2006;128:10386–10387. [PubMed: 16895402]
- Gebhardt K, Schimana J, Müller J, Fiedler H-P, Kallenborn HG, Holzenkämpfer M, Krastel P, Zeeck A, Vater J, Hölzel A, Schmid DG, Rheinheimer J, Dettner K. Screening for biologically active metabolites with endosymbiotic bacilli isolated from arthropods. *FEMS Microbiol. Lett* 2002;217:199–205. [PubMed: 12480104]
- Gehring AM, Bradley KA, Walsh CT. Enterobactin biosynthesis in *Escherichia coli*: isochorismate lyase (EntB) is a bifunctional enzyme that is phosphopantetheinylated by EntD and then acylated by EntE using ATP and 2,3-dihydroxybenzoate. *Biochemistry* 1997;36:8495–8503. [PubMed: 9214294]
- Gokhale RS, Tsuji SY, Cane DE, Khosla C. Dissecting and exploiting intermodular communication in polyketide synthases. *Science* 1999;284:482–485. [PubMed: 10205055]
- Kato Y, Bai L, Xue Q, Revill P, Yu T-W, Floss HG. Functional expression of genes involved in the biosynthesis of the novel polyketide chain extension unit, methoxymalonyl-acyl carrier protein, and engineered biosynthesis of 2-desmethyl-2-methoxy-6-deoxyerythronolide B. *J. Am. Chem. Soc* 2002;124:5268–5269. [PubMed: 11996558]
- Lambalot RH, Gehring AM, Flugel RS, Zuber P, LaCelle M, Marahiel MA, Reid R, Khosla C, Walsh CT. A new enzyme superfamily - the phosphopantetheinyl transferases. *Chem. Biol* 1996;3:923–36. [PubMed: 8939709]
- Lambalot RH, Walsh CT. Holo-[acyl-carrier-protein] synthase of *Escherichia coli*. *Methods Enzymol* 1997;279:254–62. [PubMed: 9211277]
- Ligon J, Hill S, Beck J, Zirkle R, Molnar I, Zawodny J, Money S, Schupp T. Characterization of the biosynthetic gene cluster for the antifungal polyketide soraphen A from *Sorangium cellulosum* So ce26. *Gene* 2002;285:257–267. [PubMed: 12039053]
- Ono M, Sakuda S, Ikeda H, Furihata K, Nakayama J, Suzuki A, Isogai A. Structures and biosynthesis of aflastatins: novel inhibitors of aflatoxin production by *Aspergillus parasiticus*. *J. Antibiot* 1998;51:1019–1028. [PubMed: 9918395]
- Quadri LE, Keating TA, Patel HM, Walsh CT. Assembly of the *Pseudomonas aeruginosa* nonribosomal peptide siderophore pyochelin: In vitro reconstitution of aryl-4, 2-bisthiazoline synthetase activity from PchD, PchE, and PchF. *Biochemistry* 1999;38:14941–54. [PubMed: 10555976]
- Quadri LE, Weinreb PH, Lei M, Nakano MM, Zuber P, Walsh CT. Characterization of Sfp, a *Bacillus subtilis* phosphopantetheinyl transferase for peptidyl carrier protein domains in peptide synthetases. *Biochemistry* 1998;37:1585–1595. [PubMed: 9484229]
- Reeves CD, Chung LM, Liu Y, Xue Q, Carney JR, Revill WP, Katz L. A new substrate specificity for acyl transferase domains of the ansamycin polyketide synthase in *Streptomyces hygroscopicus*. *J. Biol. Chem* 2002;277:9155–9159. [PubMed: 11786554]
- Rude MA, Khosla C. Production of ansamycin polyketide precursors in *Escherichia coli*. *J. Antibiot* 2006;59:464–470. [PubMed: 17080682]
- Santi DV, Webster RW Jr, Cleland WW. Kinetics of aminoacyl-tRNA synthetases catalyzed ATP-PPi exchange. *Methods Enzymol* 1974;29:620–627. [PubMed: 4368856]
- Schuhmann T, Vollmar D, Grond S. Biosynthetic origin of the methoxyl extender unit in bafilomycin and concanamycin using stereospecifically labeled precursors. *J. Antibiot* 2007;60:52–60. [PubMed: 17390589]
- Shoji J, Sakazaki R, Wakisaka Y, Koizumi K, Mayama M, Matsuura S. Isolation of galantins I and II, water-soluble basic peptides. Studies on antibiotics from the genus *Bacillus*. *J. Antibiot* 1975;28:122–125. [PubMed: 1112763]
- Walton LJ, Corre C, Challis GL. Mechanisms for incorporation of glycerol-derived precursors into polyketide metabolites. *J. Ind. Microbiol. Biotechnol* 2006;33:105–120. [PubMed: 16187096]
- Wenzel SC, Williamson RM, Grünanger C, Xu J, Gerth K, Martinez RA, Moss SJ, Carroll BJ, Grond S, Unkefer CJ, Müller R, Floss HG. On the biosynthetic origin of methoxymalonyl-acyl carrier protein, the substrate for incorporation of “glycolate” units into ansamitocin and soraphen A. *J. Am. Chem. Soc* 2006;128:14325–14336. [PubMed: 17076505]

- Wu K, Chung L, Revill P, Katz L, Reeves CD. The FK520 gene cluster of *Streptomyces hygroscopicus* var. *ascomyceticus* (ATCC14891) contains genes for biosynthesis of unusual polyketide extender units. *Gene* 2000;251:81–90. [PubMed: 10863099]
- Yu T-W, Bai L, Clade D, Hoffman D, Toelzer S, Trinh KQ, Xu J, Moss SJ, Leistner E, Floss HG. The biosynthetic gene cluster of the maytansinoid antitumor agent ansamitocin from *Actinosynnema pretiosum*. *Proc. Natl. Acad. Sci. USA* 2002;99:7968–7973. [PubMed: 12060743]

Figure 1.

(A) Proposed biosynthetic pathway for (2R)-methoxymalonyl-ACP formation in FK520 biosynthesis; (B) Proposed biosynthetic pathway for (2R)-methoxymalonyl-ACP formation in sorafen biosynthesis; (C) Biosynthetic pathway for (2R)-hydroxymalonyl-ACP formation in zwittermicin A biosynthesis; (D) Biosynthetic pathway for (2S)-aminomalonyl-ACP formation in zwittermicin A biosynthesis. Abbreviations: SAM, S-adenosylmethionine; SAHC, S-adenosylhomocysteine. The enzymes involved in each pathway are described in the text.

Figure 2.

Analysis of purified proteins by SDS-PAGE (12%) followed by Coomassie-blue staining. The following amounts of protein were loaded: 15 μ g ZmaD, 4 μ g ZmaE, 4 μ g ZmaG, 15 μ g ZmaH, 4 μ g ZmaI, 3 μ g ZmaJ, and 4 μ g ZmaN.

Figure 3. HPLC analysis of (A) apo- and holo-ZmaD and (B) apo- and holo-ZmaH. S denotes *Bacillus subtilis* Sfp.

Figure 4. Formation of 1,3-bisPG (A) from 3-PG by the action of 3-PGPK and (B) from G3P by the action of GAPDH.

Figure 5.

HPLC analysis of reaction mixtures containing (A) apo-ZmaD and Sfp; (B) apo-ZmaD, Sfp, and ZmaN; (C) apo-ZmaD, Sfp, ZmaN, ZmaG, and ZmaE. The arrows identify the peaks associated with the ZmaD derivatives. The letters above the peaks identify the proteins in the reaction mixture. Abbreviations: S, Sfp; N, ZmaN; E, ZmaE, G, ZmaG.

Figure 6.

HPLC analysis of ZmaH-containing reactions containing (A) apo-ZmaH and Sfp; (B) apo-ZmaH, Sfp, and ZmaJ; (C) apo-ZmaH, Sfp, ZmaJ, ZmaG, and ZmaI. The arrows identify the peaks associated with the ZmaD derivatives. The letters above the peaks identify the proteins in the reaction mixture. Abbreviations: S, Sfp; G, ZmaG; I, ZmaI; J, ZmaJ.

Table I
MALDI-TOF MS analysis of HPLC-purified ZmaD and ZmaH derivatives

Protein [*]	Theoretical [M+H] ⁺	Experimental [M+H] ⁺
ZmaD		
Apo-ZmaD	12,239	12,239
Holo-ZmaD	12,579	12,579
Glyceryl-ZmaD	12,667	12,670 [†]
Hydroxymalonyl-ZmaD	12,681	ND
Glycolyl-ZmaD [†]	12,637	12,636
ZmaH		
Apo-ZmaH	11,609	11,605
Holo-ZmaH	11,949	11,946
Seryl-ZmaH	12,037	12,038
Aminomalonyl-ZmaH	12,051	ND
Glycyl-ZmaH	12,007	12,007

Masses are in Daltons. ND, not detected.

^{*} The mass of ZmaD and ZmaH derivatives is calculated after removal of the first methionine.

[†] The mass of glyceryl-ZmaD when G3P and GAPDH are used to make 1,3-bPG.