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Syntheses of the P-Methylase Substrates of the Bialaphos Biosynthetic Pathway

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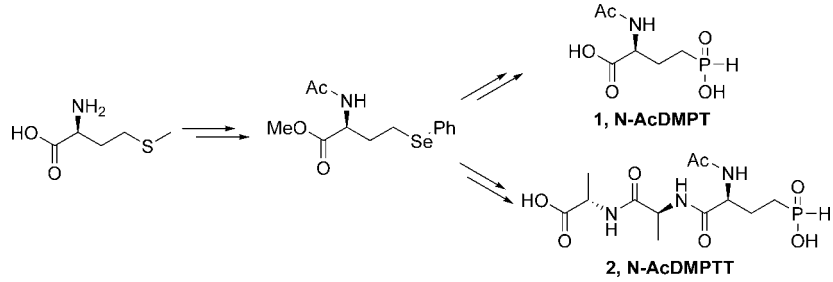
ABSTRACT



Genetic studies suggest that either *N*-acetyldemethyl phosphinothricin (1, *N*-AcDMPT) or *N*-acetyldemethyl phosphinothricin tripeptide (2, *N*-AcDMPTT) is the substrate for the P-methylation reaction in the biosynthesis of phosphinothricin tripeptide (PTT), which is widely used as an herbicide. To study the mechanism for this unique P-methylation reaction catalyzed by the BcpD protein and the functions of the unusual nonribosomal peptide synthetases involved in PTT biosynthesis, this work reports the chemical syntheses of 1 and 2.

Phosphonates are a class of organophosphorus compounds with direct C−P bond linkages. Ever since 2-aminoethylphosphonate was identified as the first naturally occurring phosphonate in 1959,[1] phosphonates have been found in many organisms.[2,3] Recently, there has been a renewed interest in natural phosphonates because most of them show important biological activities.[3,4] The currently known natural phosphonates can be roughly divided into three categories, respresented by 2-aminoethylphosphonate, K-26, and bialaphos respectively. Most known phsophonates belong to the first category, in which the C−P bond is formed by phosphoenol−pyruvate mutase catalyzed intramolecular rearrangement of phosphoenolpyruvate to phosphonopyruvate.[5−13] The phosphoenoyl−pyruvate isomerase discovered in 1988 remains as the only biochemically characterized C−P bond-forming enzyme.[5] K-26 represents the second category of natural phosphonates. K-26 contains a nonproteinogenic amino acid, (*R*)-1-amino-2-(4-hydroxylphenyl)ethylphosphonic acid (AHEP). Recent studies suggest that tyrosine is the precursor, which excludes the direct involvement of the phosphoenol−pyruvate isomerase in AHEP biosynthesis.[14,15] Phosphinothricin analine tripeptide (PTT, or bialaphos)

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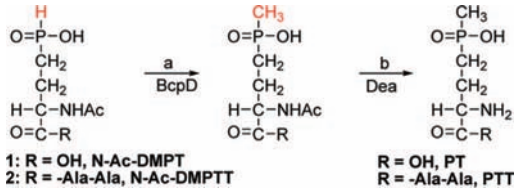
belongs to the third category. PTT is widely used in agriculture due to its bactericidal, fungicidal, and herbicidal properties.[3] PTT is unique among naturally occurring phosphonates in that it has a C−P−C bond. The first C−P bond in PTT is catalyzed by phosphoenol−pyruvate mutase, as in the first category.[3] Genetic studies suggests that a methylcobalamin dependent protein (BcpD) catalyzes the formation of the second C−P bond in PTT.[3,16−18]

Bialaphos biosynthesis in *Streptomyces hygroscopicus* and *Streptomyces viridochromogenes* has been investigated by analyzing the accumulated intermediates in the blocked PTT biosynthetic mutants.[3,19−24] It was proposed that either *N*-AcDMPT (**1**) or *N*-AcDMPTT (**2**) is the substrate for the BcpD-catalyzed P-methylation reaction (Scheme 1).[3,16−18]

Scheme 1. Proposed Last Two Steps in PTT Biosynthesis



N-AcDMPT (**1**) and *N*-AcDMPTT (**2**) are natural products with a phosphinic functional group. To date, only a very small pool of phosphinic natural products have been identified.[3] In addition, BcpD activity has not been reconstituted in vitro. To facilitate detailed mechanistic studies of this unique P-methylation reaction, convenient chemical syntheses for both *N*-AcDMPT (**1**) and *N*-AcDMPTT (**2**) are described in this paper.

The retrosynthetic analysis is outlined in Figure 1, in which both *N*-AcDMPT (**1**) and *N*-AcDMPTT (**2**) were synthesized from the same intermediate phenyl selenide **4**. The C−P bond can be constructed by coupling hypophosphite to an olefin at room temperature via a triethylborane initiated radical reaction.[25] Several methods are present in the literature on the synthesis of α-vinyl glycine.[26−28] In this paper, the

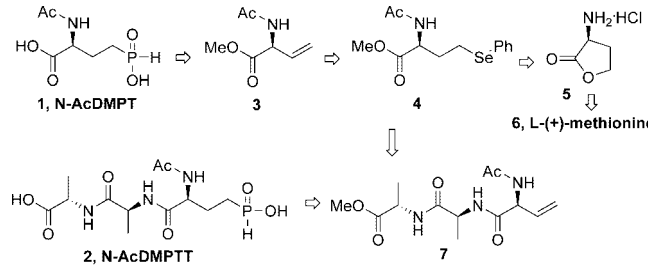
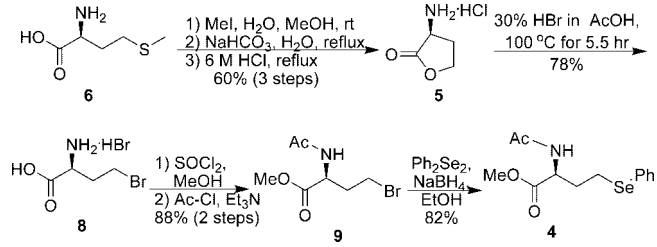


Figure 1. Retrosynthetic analysis of the *N*-AcDMPT and *N*-AcDMPTT.

oxidative elimination method from phenyl selenide **4** was chosen and conditions were optimized to resolve issues such as low yield and low optical purity.[29] One of the other intermediates, (*S*)-2-aminobutyrolactone hydrochloride (**5**) can be easily scaled up to tens of grams following a "one-pot" reaction from L-(+)-methionine.[30]

(*S*)-2-Aminobutyrolactone hydrochloride **5** was synthesized from L-(+)-methionine over three steps, including thiol-methylation with methyl iodide, hydrolysis in basic aqueous solution, and ring closure mediated by 6 M hydrochloric acid in one pot, with an overall yield of 60% (Scheme 2).[30]

Scheme 2. Synthesis of Phenyl Selenide **4**



Nucleophilic ring opening of **5** led to the formation of (*S*)-2-amino-4-bromobutyric acid hydrobromide (**8**). After recrystallization, the compound **8** obtained has an optical purity of $[\alpha]^{24}_D = -9.6$ ($c = 1.0$, H_2O), which is close to the value reported in the literature[29] ($[\alpha]^{23}_D = -6.8$ ($c = 1.0$, H_2O)). The carboxylate and amino groups in **8** were then protected sequentially by methylation and acetylation to form (*S*)-methyl 2-acetamido-4-bromobutanoate (**9**). The conversion of bromide **9** to phenyl senelide **4** was achieved following the standard Sharpless conditions using sodium phenylselenide generated from diphenyldiselenide and sodium borohydride in situ.[31]

The production of α-vinyl glycine **3** from phenyl selenide **4** was the most challenging step in compound **1** synthesis.

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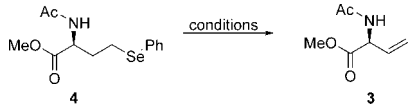
Both hydrogen peroxide and sodium periodate were examined as the oxidants. The results are summarized in Table 1.

Table 1. One-Pot Oxidative Elimination of Phenyl Selenide **4**[a]



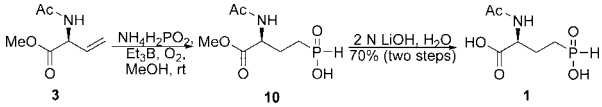
entry	oxidant	solvent	T (°C)	time (h)	yields[b] (%)
1	30% H_2O_2	H_2Cl_2	reflux	5	28
2	30% H_2O_2	CH_2Cl_2	reflux	24	30[c]
3[d]	30% H_2O_2	CH_2Cl_2	reflux	5	33[c]
4	$NaIO_4$	THF/H_2O	rt	5	37
5[e]	$NaIO_4$	THF/H_2O	rt	5	30[c]
6	$NaIO_4$/SiO_2	CH_2Cl_2	reflux	5	70
7	$NaIO_4$/SiO_2	CH_2Cl_2	reflux	10	85

[a] All reactions were performed with oxidant 30% H_2O_2 (5 equiv) or $NaIO_4$ (2 equiv) in solvent with or without additive in proper temperature or reaction time. [b] Isolated yield. [c] The product contained unseparated impurity. [d] With the additive pyridine. [e] With the additive $NaHCO_3$.

A similar oxidative elimination reaction has been reported in the literature with up to 83% yield when 30% H_2O_2 was used as the oxidant.[29] However, the oxidative elimination reaction from phenyl selenide **4** was low in yield following similar conditions as those in literature (entry 1, Table 1).[29] Extending the reaction time to 1 day resulted in no noticeable difference (entry 2, Table 1). Organic base pyridine was reported to accelerate this reaction;[29] however, the addition of pyridine did not improve the yield either. Similar results were obtained when inorganic base $NaHCO_3$ was used (entries 3 and 5, Table 1). Another oxidant, $NaIO_4$, was also examined in a THF/H_2O solvent system, in which a slightly higher yield was obtained (entry 4). Interestingly, when the silica gel supported $NaIO_4$ was used in the presence of a trace amount of water, the oxidative elimination was readily achieved. Under optimized conditions, the phenyl selenide **4** was synthesized from **3** in 85% yield (entry 6−7, Table 1).

After the key intermediate **3** was obtained, triethylborane-initiated radical reaction was explored to couple hypophosphite and α-vinyl glycine **3** to form the C−P bond-containing compound **10** (Scheme 3).[25] The coupling reaction was

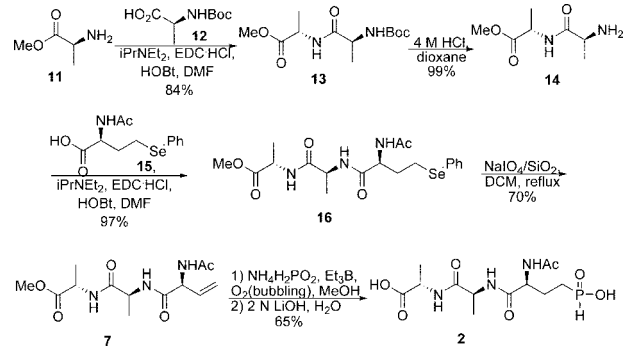
Scheme 3. Synthesis of *N*-AcDMPT (**1**)



almost quantitative when monitored by ^{31}P NMR. After the desired product **10** was extracted into the aqueous phase, it was then treated with 2 N LiOH to deprotect the carboxylate. The desired product, *N*-AcDMPT (**1**), was produced in 70% yield in the ammonium form after cation exchange chromatography and HPLC purification (Scheme 3).

With the success of *N*-AcDMPT (**1**) synthesis, the tripeptide *N*-AcDMPTT (**2**) was also synthesized using phenyl selenide **4** as the key intermediate. The dipeptide (Ala-Ala) **13** was produced from the amino and carboxylate-protected alanine **11** and **12** by following a standard peptide synthetic procedure using EDC (1-[3-(dimethylaminopropyl)]-3-ethylcarbodiimide), HOBt (1-hydroxybenzotriazole hydrate), and Et_3N as the reagents (Scheme 4). The same method was

Scheme 4. Synthesis of *N*-AcDMPTT (**2**)



then used for the construction of the second peptide bond using *N*-terminal deprotected dipeptide **14** and the carboxylate-deprotected phenyl selenide **15**. The α-vinyl tripeptide **7** was produced by the oxidative elimination of tripeptide phenyl selenide **16** in 70% yield under the same conditions as those optimized for compound **4** (Table 1).

The coupling reaction between hypophosphite and α-vinyl glycine containing tripeptide **7** in compound **2** synthesis was proven to be more difficult than in compound **1** synthesis. Under the same conditions used for compound **1** synthesis, the coupling reaction for α-vinyl glycine containing tripeptide **7** and hypophosphite was very slow. Almost no detectable conversion occurred in 3 h when monitored by ^{31}P NMR. However, if air bubbling was introduced and 1 equiv of Et_3B was added every 2 h, the rate for the coupling reaction dramatically increased and the coupling reaction finished within 8 h as revealed by ^{31}P NMR. Overall, after C−P bond formation, deprotection of the carboxylate group, and HPLC purification, compound **2** was obtained in 65% yield over the last two steps (Scheme 4).

In summary, we describe the syntheses of two phosphinic acids, *N*-AcDMPT (**1**) and *N*-AcDMPTT (**2**), from readily available L-(+)-methionine using the same key intermediate, phenyl selenide **4**. With these two compounds available, we are now ready for mechanistic studies on the unique nonribosomal peptide synthetases and cobalamin-dependent P-methylase in PTT biosynthesis.

BcpD, one of the tailoring enzymes in PTT biosynthesis, belongs to a large family of proteins initially identified by bioinformatics as the radical *S*-adenosylmethionine (SAM)

superfamily.[32] According to a more recent survey, this family of enzymes has expanded from the initial 600 members to more than 2800 members.[33] To date, none of the members of the methylcobalamin subfamily have been characterized biochemically.

Before any detailed mechanistic studies can be carried out, the most critical issue to be resolved is the substrate identities for both BcpD and the three nonribosomal peptide synthetases in PTT biosynthesis. Several different approaches are employed by nonribosomal peptide synthetases to incorporate nonproteinogenic amino acids into their products.[34] First, nonribosomal peptide synthases can incorporate domains in *cis* into the nonribosomal peptide assembly lines. These domains can then introduce the necessary modifications to produce the nonproteinogenic amino acids. Second, nonribosomal peptide gene clusters can encode the tailoring enzymes as individual proteins, which utilize the peptide tethered to the terminal thiol of phosphopantetheinyl arm of the acyl carrier protein as the substrate. Third, after the nonribosomal peptides are assembled and released from the assembly line by the action of thioesterase, they undergo further tailoring by dedicated enzymes. Based on the above general rules governing the nonribosomal peptide assembly, the following scenarios need to be considered for the BcpD-catalyzed P-methylation reaction in PTT biosynthesis: (a) BcpD utilizes *N*-AcDMPT (**1**) as the substrate and the methylated product *N*-acetyl phosphinothricin is then utilized as the substrate for the nonribosomal peptide synthetase; (b) *N*-AcDMPT (**1**) is loaded onto the acyl-carrier domain of the nonribosomal peptide synthetase and the substrate tethered acyl carrier protein serves as the substrate for BcpD; (c) after the tripeptide, *N*-AcDMPTT (**2**), is assembled and released from the nonribosomal peptide synthetase by a thioesterase, it is then methylated by BcpD.

The PTT biosynthetic cluster has three nonribosomal peptide synthetase genes (*phsA*, *phsB*, and *phsC*). PhsA only has an adenylation domain and a peptide carrier protein domain and is proposed to be responsible for activating the first amino acid (*N*-AcDMPT) in PTT biosynthesis. According to the above BcpD scenarios, the substrate for PhsA also needs to be clarified. More interestingly, PhsA has a unique *N*-terminal thioesterase domain.[34,35] This is unusual because the thioesterase domain generally is located at the *C*-terminal of the last module of nonribosomal peptide synthetase rather than the first module.[36] When the PhsA *N*-terminal domain is deleted, it abolishes *S. viridochromogenes* PTT biosynthesis, which highly suggests its importance in PTT biosynthesis. With compounds **1** and **2** in hand, the substrate identities for both PhsA and BcpD will be examined in the near future.

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Supporting Information Available: Procedures and characterization spectra for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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