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Supplementary Material Available: Experimental procedures and spectral data (5 pages). Ordering information is given on any current masthead page.

Synthesis of the Unusual Metabolite Carboxyphosphonoenolpyruvate. Cloning and Expression of Carboxyphosphonoenolpyruvate Mutase

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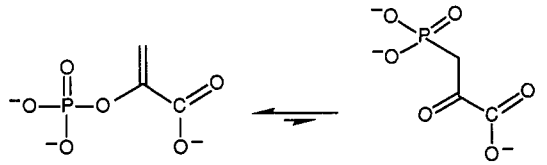
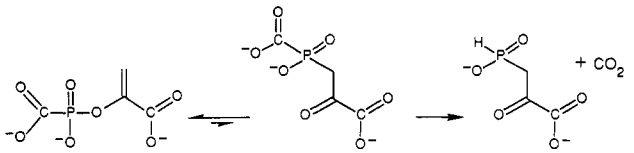
The carbon-phosphorus bond of most naturally occurring phosphonates derives from a 1,3-phospho group transfer reaction between phosphoenolpyruvate (**1**) and phosphonopyruvate (**2**).[1] The equilibrium for this rearrangement, which is catalyzed by phosphoenolpyruvate mutase (EC 6.4.2.9), lies predominantly toward **1**.[1] This enzyme is not, however, responsible for the



1 **2**

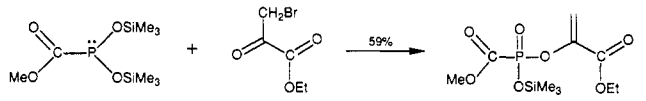
formation of either of the two carbon-phosphorus bonds in bialaphos, a powerful herbicide isolated from *Streptomyces hygroscopicus*. Thus Seto and his collaborators[2,3] have shown that the biosynthetic pathway to bialaphos involves carboxyphosphonoenolpyruvate mutase (CPEP mutase), which catalyzes the formation of (hydroxyphosphinyl)pyruvate (**5**) from carboxyphosphonoenolpyruvate (**3**, CPEP). By analogy with phosphoenolpyruvate mutase, the first step of the CPEP mutase reaction presumably generates the new carbon-phosphorus bond by carboxyphospho group migration to give **4**. This reaction seems likely



3 **4** **5**

to be energetically unfavorable (as is the conversion of **1** to **2**). In the second step, the intermediate **4** would decarboxylate, thus driving the reaction toward **5**. Mechanistic studies on this interesting enzyme will be possible only with a supply of the substrate **3**, a continuous product assay for **5**, and ready access to the enzyme. We report here the chemical synthesis of the unusual phosphonate **3**, the cloning of the mutase gene from *S. hygroscopicus* and its expression at high levels in *Escherichia coli*, and a convenient assay for the product, **5**.

Despite earlier suggestions that **3** is "extremely unstable",[3] the chemical synthesis of **3** proceeded smoothly, the key step[4] involving a Perkov reaction between bis(trimethylsiloxy)(methoxycarbonyl)phosphine[5] (**6**) and ethyl bromopyruvate to give the triester **7**. Although recent studies on the hydrolysis of trialkyl



6 **7**

esters of phosphonoformate have shown that the carbon-phosphorus bond is readily cleaved by attack at carbonyl,[6] treatment of **7** with aqueous base first removes the reactive $SiMe_3$ group to give the diester, in which the carbon-phosphorus bond is much less vulnerable. The triester **7** was readily converted to **3** by the careful addition of 3 equiv of aqueous NaOH.[7] The spectroscopic data[7] were consistent with those reported for the natural product.[2] The trisodium salt of **3** is stable in water over several days at room temperature. Carboxyphosphonoenolpyruvate (**3**) will be useful for studies of both CPEP mutase and the enzyme that catalyzes CPEP formation from phosphoenolpyruvate and phosphonoformate.[8]

To develop an assay for CPEP mutase, a sample of **5**[9,10] was prepared by the transamination of (hydroxyphosphinyl)alanine[11] with glyoxylic acid-$Cu(OAc)_2$.[12] We had earlier shown that **2** is a relatively poor substrate for malate dehydrogenase (MDH), having a high K_m of 11 mM. In terms of both size and charge, **5** should be a better mimic for oxaloacetate than **2**. As predicted, **5** is a good substrate for MDH, having a K_m of 0.68 mM and a k_{cat} of 164 s^{-1}.[13] Interestingly, the product of this reaction, (hydroxyphosphinyl)lactate, has been found in extracts of *S. hygroscopicus*.[14] Using MDH/NADH, therefore, a continuous coupled enzyme assay can be established for CPEP mutase.

Using the partial gene sequence reported by Hidaka et al.,[2] we have cloned the gene for CPEP mutase from *S. hygroscopicus* into *E. coli*. The open reading frame encodes a protein of 295 amino acids, the calculated molecular weight of which (32 700) agrees with that determined for the purified enzyme (32 000 ± 1000).[2] The gene was expressed in *E. coli* using the T7 pET11 vector, which produced CPEP mutase at 20% of the total cell

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(7) Addition of methanol to the aqueous solution caused precipitation of trisodium carboxyphosphonoenolpyruvate (**3**) in 53% yield: mp 275-280 °C dec; ^1H NMR (250.1 MHz, D_2O) δ 5.54 (t, $J_{PH} = J_{gem} = 1.7$ Hz, 1 H), 5.20 (t, $J_{PH} = J_{gem} = 1.8$ Hz, 1 H); ^{13}C NMR (62.9 MHz, D_2O) δ 179.6 (d, $J_{PC} = 235.4$ Hz, $PCOO^-$), 173.4 (d, $J_{PC} = 6.1$ Hz, $CCOO^-$), 151.8 (d, $J_{PC} = 9.2$ Hz, $C{=}CH_2$), 106.0 (d, $J_{PC} = 4.8$ Hz, $C{=}CH_2$); ^{31}P NMR (101.3 MHz, D_2O) δ -1.77 (s); IR (KBr) 1603 cm^{-1}. Calcd for $C_4H_2O_7PNa_3$: C, 18.34; H, 0.77; P, 11.82. Found: C, 18.00; H, 1.01; P, 11.48.

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(10) **5** was purified by ion-exchange chromatography (on AG1-8X, formate form, eluting with triethylammonium bicarbonate buffer, then on Dowex-50, sodium form, eluting with water) to give **5** in 30% yield: ^1H NMR (500 MHz, H_2O) δ 7.33 (dt, $J_{PH} = 550.3$, $J_{HH} = 1.7$ Hz, 1 H), 3.37 (dd, $J_{PH} = 18.9$, $J_{HH} = 1.7$ Hz, 2 H); ^{13}C NMR (100.6 MHz, H_2O) δ 199.9 (br s, $CH_2C(O)$), 168.9 (s, COO^-), 46.7 (d, $J_{PC} = 71.7$ Hz, PCH_2); ^{31}P NMR (121.5 MHz, H_2O) 17.7 ppm (dt, $J_{PH} = 550.3$, $J_{PH} = 18.9$ Hz, ^1H coupled); MS (negative-ion FAB, glycerol matrix) m/z 172.9623 (172.9616 calcd for $C_3H_3NaO_5P$, M - Na).

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(13) Malate dehydrogenase (0.56 μg) was added to a solution containing 50 mM MES buffer, pH 6.5 (1.0 mL), NADH (0.1 mg), **5** (0.038-7.7 mM) at 30 °C. The A_{340nm} was monitored with time. The k_{cat} was calculated using 37 000 as the molecular weight for MDH: Wolfenstein, C.; Englard, S.; Listowsky, I. *J. Biol. Chem.* **1969**, *244*, 6415. The K_m of oxaloacetate is 4 μM at pH 6.4: Cassman, M.; Englard, S. *J. Biol. Chem.* **1966**, *241*, 793.

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protein. CPEP mutase was readily purified to homogeneity.[3,15] Using synthetic substrate and the coupled enzyme assay, carboxyphosphonoenolpyruvate has a K_m of 0.27 mM and a k_{cat} of 0.020 s^{-1} in the mutase reaction.[16] The low k_{cat} may derive from the fact that the carboxyphospho group transfer involved in the conversion of **3** to **4** is highly endergonic. In qualitative agreement with the observation of Hidaka et al.,[3] CPEP mutase is more than 10 times as active in the presence of Mn(II) as in the presence of Mg(II). We currently aim to establish whether the presumed rearrangement of **3** to **4** proceeds via a carboxyphospho-enzyme intermediate similar to that suggested for the interconversion of **1** and **2**,[17] and whether the decarboxylation of **4** to **5** is enzyme catalyzed.

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(15) CPEP mutase was purified by an ammonium sulfate precipitation, followed by chromatography on hydroxyapatite [eluting with potassium phosphate (10–200 mM), pH 6.5], and then Mono Q 10:10 [eluting with NaCl (0–800 mM) in 50 mM Tris·HCl buffer, pH 7.5].

(16) CPEP mutase (50 μg, 1.85×10^{-3} unit) was added to a solution containing 50 mM MES buffer, pH 6.5 (0.48 mL), 0.1 M $MnCl_2$ (10 μL), NADH (50 μg), malate dehydrogenase (14 μg), and carboxyphosphonoenolpyruvate (0.038–3.8 mM) at 30 °C. The consumption of NADH was monitored at 340 nm.

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New Carbohydrate-Based Materials for the Stabilization of Proteins

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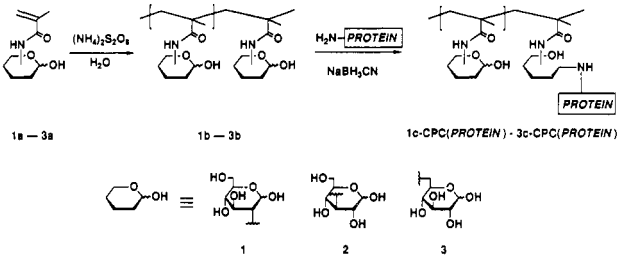
We report here the synthesis of a series of new carbohydrate-based materials and their use for the stabilization of proteins.[1] We prepared a series of aminoglucose-based monomers, **1a–3a**, by reaction of the appropriate amine with methacryloyl chloride in methanol. Treatment of **1a–3a** with ammonium persulfate in water at temperatures from 5 to 70 °C gave the

Scheme I



carbohydrate-based macromolecules **1b–3b** in yields of >80%. These water-soluble materials contain a high density of masked aldehyde functionality and have absolute molecular weights of $>4 \times 10^6$ daltons (Da) with polydispersities <1.4.[2,3] Incubation of macromolecules **1b–3b** with the desired protein and sodium cyanoborohydride in borate buffer (pH 8–9) at 37 °C gave carbohydrate–protein conjugates (CPC) of proteases [α-chymotrypsin [CPC(CT)], trypsin [CPC(Try)], and subtilisin BPN′ [CPC(BPN′)]], an endonuclease [CPC(*Eco*RI)], and an antibody that binds aldrin [CPC(M_{ab} 8H11)] (Scheme I).[4,5] Amino acid analysis of the CPC(proteases) found that approximately three to six lysines of each protein are conjugated to the carbohydrate-based macromolecule.[6] We found that the CPC(proteases) and the native enzymes have similar kinetic parameters (k_{cat} and K_m).[7-12]

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(5) The **1**-CPC(protein)–**3**-CPC(protein) materials were purified by gel filtration chromatography using 0.05 M sodium borate solution at pH 8 on Sephacryl HR-200 at a flow rate of 1.5 mL/min. Alternatively, isolation by dialysis of the reaction solution using Spectra Por CE 100K MWCO membrane against 2 × 500 mL of 0.05 M sodium borate at pH 8 for approximately 48 h gave white powders that could be stored indefinitely at room temperature without loss of activity with approximately 40% yields for α-chymotrypsin (EC 3.4.21.1, Sigma) and trypsin (EC 3.4.21.4, Sigma) conjugates and approximately 10% yields (60% recovered activity) for subtilisin BPN′ (type XXVII, Sigma) conjugates. **1c**-CPC(M_{ab} 8H11) was purified by gel filtration chromatography using 0.02 M sodium phosphate and 0.05 M sodium chloride solution at pH 7 on Sephacryl HR-300 at a flow rate of 0.5 mL/min. The protein concentration was determined by measurement of the optical density at 280 nm. **1c**-CPC(*Eco*RI) was purified by gel filtration chromatography using 0.02 M sodium borate solution at pH 8 on Sephacryl HR-200 at a flow rate of 2.5 mL/min.

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(7) The kinetic parameters (k_{cat}, K_m) for the native enzymes and the CPC analogues are as follows:[8-12] α-chymotrypsin (40 s^{-1}, 33 μM), **1c**-CPC(CT) (46 s^{-1}, 20 μM); trypsin (760 s^{-1}, 0.90 μM), **1c**-CPC(Try) (890 s^{-1}, 1.2 μM); subtilisin BPN′ (240 s^{-1}, 83 μM), **1c**-CPC(BPN′) (350 s^{-1}, 76 μM).

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