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Studies on the Biodegradation of Fosfomycin: Synthesis of ^{13}C-Labeled Intermediates, Feeding Experiments with *Rhizobium huakuii* PMY1, and Isolation of Labeled Amino Acids from Cell Mass by HPLC

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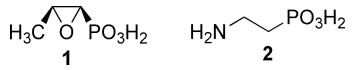
Abstract: Racemic (1*R**,2*R**)-1,2-dihydroxy-[1-$^{13}C_1$]propylphosphonic acid and 1-hydroxy-[1-$^{13}C_1$]acetone were synthesized and fed to *R. huakuii* PMY1. Alanine and a mixture of valine and methionine were isolated as their *N*-acetyl derivatives from the cell hydrolysate by reversed-phase HPLC and analyzed by NMR spectroscopy. It was found that the carbon atoms of the respective carboxyl groups were highly ^{13}C-labeled (up to 65%). Hydroxyacetone is therefore considered an obligatory intermediate of the biodegradation of fosfomycin by *R. huakuii* PMY1.

Keywords: amino acids · biotransformations · fosfomycin · hydroxyacetone · isotopic labeling

Introduction

Fosfomycin (**1**) is one of the few natural products to contain a chemically very stable P–C bond.[1] It is produced by strains of *Streptomyces* and *Pseudomonas syringae* and is utilized as a clinical antibiotic that blocks[2] the formation of bacterial cell walls.



1 **2**

Elucidation of the fosfomycin biosynthetic pathway, which comprises five metabolic steps (starting from phosphoenolpyruvate), has proved challenging, since three of these steps involve extraordinary chemical transformations.[3–7] Meanwhile, its mineralization remains largely unexplored, as is the case for the majority of man-made and biogenic phosphonates, with the exception of 2-aminoethylphosphonic acid (**2**). Acquisition of adequate supplies of inorganic phosphate (P_i), as the preferred phosphorus source, is vital for any living cell. Under conditions of phosphorus starvation, gene systems are induced, the products of which are involved in the acquisition and assimilation of phosphorus from a range of organic phosphorus substrates, including phosphonates. For the most part, microorganisms use phosphonates only as a phosphorus source, with this breakdown being strictly regulated by the *pho* regulon via the P_i released from the phosphonate substrate. Some bacteria, however, produce P–C bond-cleaving enzymes (P–C hydrolases), which are not subject to control by P_i, thereby facilitating microbial utilization of the phosphonate as a phosphorus, carbon (and nitrogen), and energy source.[3,8] Some years ago, we reported the isolation of the bacterium *Rhizobium huakuii* PMY1 using fosfomycin at 10 mM as a carbon or carbon and phosphorus source with concomitant P_i release.[9]

More recently, we demonstrated that of the four stereoisomeric 1,2-dihydroxypropylphosphonic acids only the (1*R*,2*R*)-isomer supported growth, as evidenced by the release of P_i.[10] None of the salts of (±)-1,2-epoxybutyl-, (±)-1,2-dihydroxyethyl-, 2-oxopropyl-, (*S*)-2-hydroxypropyl-, (±)-1-hydroxypropyl-, or (±)-1-hydroxy-2-oxopropylphosphonic acid supported growth. In vitro P–C bond cleavage activity was detected only for the latter phosphonic acid. *R. huakuii* PMY1 grew well on (*R*)- and (*S*)-lactic acid and hydroxyacetone, but less well on propionic acid, and not on acetone or (*R*)- and (±)-1,2-propanediol.[10] Furthermore, phosphite is not involved in fosfomycin biodegradation since it cannot be used as a phosphorus source by *R. huakuii* PMY1, and no phosphite oxidase activity could be detected in PMY1 cell-free extracts.[10] These results indicate that the enzymes involved in the biodegradation of fosfomycin are very specific and that the first step is a formal hydrolytic ring opening. Most probably, a glycol monoester[11] is

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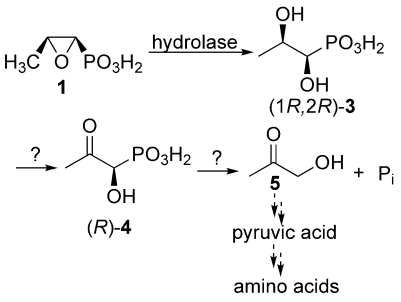


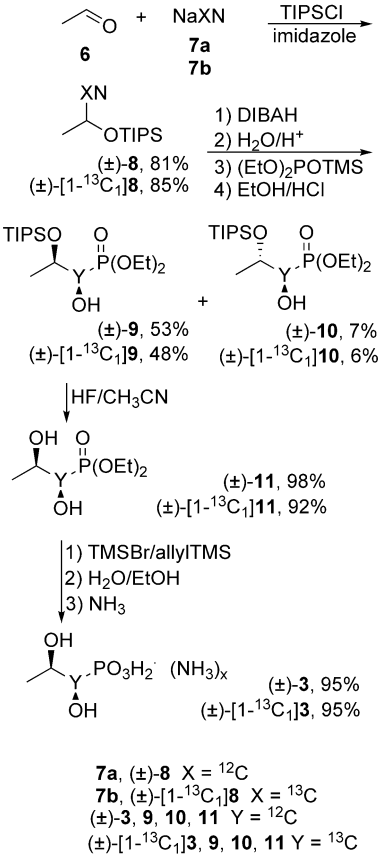
Scheme 1. Putative pathway for the biodegradation of fosfomycin.

formed as an enzyme-bound intermediate, which is hydrolyzed to (1*R*,2*R*)-**3** (see Scheme 1), the stereochemical outcome of which is net inversion of the configuration at C-2. Interestingly, FosX proteins from *M. loti* and *Listeria* catalyze the metal-ion-assisted ring opening of fosfomycin by water at C-1, giving the (1*S*,2*S*)-diol, as a genomic encoded mechanism of resistance.[12]

Results and Discussion

To date, our studies on the mineralization of fosfomycin by cells of *R. huakuii* PMY1 have focused on the P moiety by quantifying P_i release into the culture medium; fosfomycin consumption during microbial growth has also been monitored by GC/MS analysis.[9] However, as yet we have no information as to what happens to the three-carbon unit of fosfomycin during degradation, and whether hydroxyacetone and lactic acid are obligatory intermediates in this pathway (Scheme 1). We reasoned that it would be possible to address these points through two complementary feeding experiments starting with (1*R*,2*R*)-1,2-dihydroxy-[1-$^{13}C_1$]propylphosphonic acid (**3**). As fosfomycin and all obligatory intermediates of the biodegradation are used as carbon and energy sources, part of them will be incorporated into cellular components. We assume that the phosphorus is cleaved from a three-carbon-atom portion and that the functionalized propane is further transformed to pyruvic acid, which is incorporated into amino acids. We planned to hydrolyze the harvested cells, after feeding, with 6 M HCl and to isolate the labeled amino acids, possibly after derivatization, by an HPLC method. NMR spectroscopy of the fractions obtained would allow determination of the location and quantification of the incorporated ^{13}C atoms from the precursors.

Synthesis of ^{13}C-labeled 1,2-dihydroxypropylphosphonic acid: The synthesis of homochiral (1*R*,2*R*)-1,2-dihydroxy-[1-$^{13}C_1$]propylphosphonic acid is quite difficult. We opted for the much more easily accessible racemic mixture, as we had established for the unlabeled series that the (1*S*,2*S*)-enantiomer does not interfere with the biodegradation of the (1*R*,2*R*)-enantiomer by *R. huakuii* PMY1.[10] The synthesis as outlined in Scheme 2 was built on the known addition of



Scheme 2. Synthesis of unlabeled and ^{13}C-labeled (1*R**,2*R**)-1,2-(±)-dihydroxypropylphosphonic acids **3**.

diethyl trimethylsilyl phosphite to protected lactaldehyde and was optimized in the unlabeled series.[10]

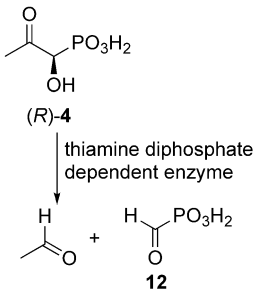
The acetaldehyde cyanohydrin formed as an intermediate in a mixture of DMF, acetaldehyde, NaCN, imidazole, and TIPSCl (TIPS = triisopropylsilyl) was silylated immediately and the product was isolated in 81% yield after 60 h. Reduction of the nitrile (±)-**8** in dry toluene, followed by acidic work-up to hydrolyze the imine, furnished the crude TIPS-protected lactaldehyde, which was used immediately without further purification.[13] Diethyl trimethylsilyl phosphite was added to a solution of the aldehyde in dry toluene at −78 °C and then the mixture was allowed to warm to 10 °C.[10] Removal of the volatile components under reduced pressure and selective monodesilylation with HCl in dry ethanol yielded a mixture of racemic diastereomers, which were separated by flash chromatography. The predominating and less polar isomer (±)-**9**, with the relative (1*R**,2*R**) configuration as deduced from the configuration of (±)-**3**, was obtained in 53% yield, whereas the more polar (±)-**10** with the (1*R**,2*S**) configuration was obtained in 7% yield. The desired isomer (±)-**9** was deprotected in two steps, first with HF in CH_3CN/H_2O in 98% yield, and then with TMSBr/allylTMS. The free phosphonic acid was converted into the ammonium salt, which was identical by NMR spectroscopic analysis to an authentic sample of the ammonium salt of

(1*R*,2*R*)-1,2-dihydroxypropylphosphonic acid derived from fosfomycin.[10] The ^{13}C-labeled species (±)-[1-$^{13}C_1$]**3** was accessed analogously in similar yield, except that NaCN was replaced by commercially available Na^{13}CN (99% ^{13}C).

Feeding experiments and isolation of amino acids: Feeding experiments with labeled precursors are very useful and a standard methodology in biosynthetic studies. The natural products formed are isolated and investigated for the incorporation of the label. However, elucidation of the mineralization pathway of a natural product or a xenobiotic is more complicated, as the degradative intermediates, on their way to water and CO_2 as the final metabolic products, are rarely detected. Knowing that fosfomycin is used as a carbon and energy source by cells of *R. huakuii* PMY1, we reasoned that isolation of labeled amino acids biosynthesized from its degradation product(s) could help to deduce the biodegradation pathway.

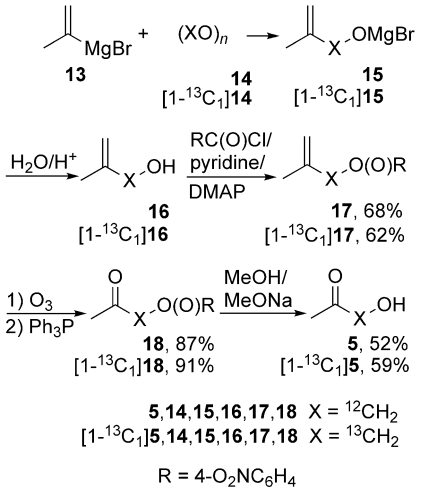
Cells of *R. huakuii* PMY1 were grown in minimal salts medium with phosphate-free yeast extract, but omitting the essential amino acids used previously, supplemented with undiluted 1,2-dihydroxypropylphosphonic acid (±)-[1-$^{13}C_1$]**3** (7.5 mM) as the sole phosphorus, carbon, and energy source.[9] After 10 days of growth at 30°C on an orbital shaker at 100 rpm, 2.2 mM P_i had been released into the culture medium. No spontaneous P_i release was observed either in uninoculated control experiments or from cultures incubated in the absence of substrate. Cells were harvested at 10000×*g* for 15 min at 4°C and washed once in 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)/NaOH buffer (pH 7.0).

The sterilized cell suspension was hydrolyzed in 6M HCl and the liberated amino acids were N-acetylated by reaction with acetic anhydride. This derivatization and the use of reversed-phase HPLC for the isolation of *N*-acetyl amino acids were necessary, since preliminary experiments had shown that alanine isolated by HPLC as its hydrochloride was too impure by ^{1}H NMR spectroscopy (600.13 MHz). It had to be purified a second time, but there were still impurities present. Furthermore, reversed-phase HPLC additionally allowed the isolation of a mixture of *N*-acetylvaline and *N*-acetylmethionine. Finally, the cells grown on the C-1 ^{13}C-labeled 1,2-dihydroxypropylphosphonic acid were worked-up and gave *N*-acetylalanine (0.4 mg) and a mixture of *N*-acetylvaline and *N*-acetylmethionine (0.3 mg, ratio 1:0.26 by ^{1}H NMR) in sufficient purity for investigation by NMR spectroscopy (^{1}H and ^{13}C NMR at 600.13 and 150.90 MHz, respectively) in D_2O. For comparison, commercially available reference samples were used for HPLC and NMR spectroscopy. The alanine was labeled at C-1 (52%, from the height of the signals due to 3-H at $\delta = 1.32$ ppm, doublet with $J = 7.3$ Hz, flanked by satellite doublets with $J(^{13}C,H) = 4.3$ Hz). The 2-H signal ($\delta = 4.18$ ppm) was composed of a quartet ($J = 7.3$ Hz) due to the unlabeled species, overlapping with a doublet of quartets ($J = 7.3$ and 5.0 Hz) due to the ^{13}C-labeled species. On ^{13}C decoupling, the 2-H signal collapsed to a quartet with $J = 7.3$ Hz. The C-1 resonance was the strongest signal in the ^{13}C NMR spectrum. The C-1 position of *N*-acetylvaline was labeled (23% ^{13}C), as evidenced by the doublet ($J = 5.8$ Hz) at $\delta = 4.14$ ppm flanked by doublets ($J = 5.8$ Hz) in the ^{1}H NMR spectrum, which collapsed to a doublet upon ^{13}C decoupling. Furthermore, the carboxyl signal was strongly enhanced in the ^{13}C NMR spectrum. The methionine was essentially unlabeled at any position. These findings show that the three-carbon-atom fragment of fosfomycin stays intact as long as it is attached to the phosphorus atom. The P–C bond cleavage releases a propane derivative of unknown substitution pattern, possibly hydroxyacetone. Propane-1,2-diol has been excluded as an intermediate of the biodegradation pathway of fosfomycin.[10] If 2-oxophosphonic acid (*R*)-**4** (Scheme 1) is an obligatory intermediate and not just a shunt metabolite, an alternative pathway for its degradation could be envisaged (Scheme 3). A thiamine diphosphate-dependent enzyme could degrade it to acetaldehyde and formylphosphonic acid (**12**), which would be further metabolized. The mechanism for this C–C bond breaking is somewhat reminiscent of the transketolase-catalyzed reaction of the Calvin cycle.[14] However, this cleavage is not compatible with the above results.



Scheme 3. Alternative pathway for the biodegradation of 2-oxopropylphosphonic acid (*R*)-**4**.

Synthesis and feeding of 1-hydroxy-[1-$^{13}C_1$]acetone: If hydroxyacetone is a degradation product of fosfomycin, its C-1-labeled species should give the same labeling pattern for alanine and valine as 1,2-dihydroxy-[1-$^{13}C_1$]propylphosphonic acid. To test this hypothesis, ^{13}C-labeled hydroxyacetone was prepared after optimizing the reaction sequence in the unlabeled series (Scheme 4).



Scheme 4. Synthesis of labeled and unlabeled hydroxyacetone.

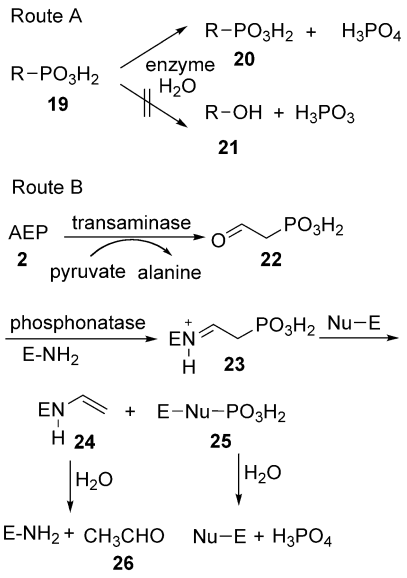
Isopropenylmagnesium bromide was treated with paraformaldehyde to give 2-methyl-2-propenol (**16**), which was not purified because of its low boiling point, but directly esterified with 4-nitrobenzoyl chloride/pyridine to give *p*-nitrobenzoate **17** in 68% yield. The 3,5-dinitrobenzoyl group was also tested as a protective group to facilitate the handling of this small molecule, but was found to be less well suited for the requisite steps than the 4-nitrobenzoyl group. The ester **17** was ozonolyzed and then treated with Ph_3P as a reductant to yield the protected hydroxyacetone **18** as a crystalline product in 87% yield. It was transesterified with NaOMe in MeOH, and the hydroxyacetone (**5**) formed was obtained in 53% yield after purification by flash chromatography and distillation. The ^{13}C-labeled isotopomer [1-$^{13}C_1$]**5** was prepared by the same steps in similar yield.

Cells of *R. huakuii* PMY1 were grown on minimal salts medium supplemented with labeled hydroxyacetone (5 mM) as the sole source of carbon and energy; 1 mmol L^{-1} of KH_2PO_4 was added as a phosphorus source. The cells were worked-up as before and *N*-acetylalanine and a mixture of *N*-acetylvaline and *N*-acetylmethionine (ratio 1:0.33) were isolated by reversed-phase HPLC. Alanine and valine were found to be ^{13}C-labeled at C-1, 52 and 65%, respectively. Methionine was not labeled by [1-$^{13}C_1$]**5**. These data are taken as proof that hydroxyacetone is an obligatory intermediate in the biodegradation of fosfomycin.

Two pathways have been proposed for the degradation of hydroxyacetone. The first involves oxidation via methylglyoxal to pyruvate, and is catalyzed by acetol dehydrogenase [EC 1.1.1.-] and methylglyoxal dehydrogenase [EC 1.2.1.23].[15] In the second pathway, C–C cleavage of hydroxyacetone via an acetol monooxygenase gives rise to acetate and a C_1-compound, possibly formaldehyde.[16] The results with the ^{13}C-labeled compound showed that the first pathway must be operative in *R. huakuii* PMY1. Furthermore, acetol dehydrogenase and methylglyoxal dehydrogenase activities of 6.2 U per mg of cell-extract protein and 6.7 U per mg of cell-extract protein, respectively, were detected in extracts of *R. huakuii* PMY1 cells grown on fosfomycin (5 mM) as a phosphorus, carbon, and energy source. No acetol monooxygenase activity could be detected.

As the growth medium does not contain sufficient amounts of alanine and valine, but evidently sufficient methionine, the former two have to be biosynthesized from pyruvate formed in two steps from hydroxyacetone.[14] It is transaminated from glutamate to give alanine. Two molecules of pyruvate are condensed with loss of CO_2 to form α-acetolactic acid, which is converted in three steps to α-ketoisovaleric acid. This is transaminated to valine. If pyruvic acid ^{13}C-labeled at C-1 is used, valine ^{13}C-labeled at C-1 will result, in agreement with our results.

Enzymatic cleavage of P–C bonds in phosphonates **19** studied so far furnished orthophosphate and organic compounds **20**, with an H–C bond in place of the P–C bond (Scheme 5, route A).[8] This formal hydrolytic process reduces the carbon atom and oxidizes the phosphorus atom. No enzyme has been found that effects formation of phosphite and an alcohol **21**, which would also be compatible with hydrolytic cleavage of a P–C bond. From a mechanistic point of view, cleavage of the P–C bond in (*R*)-1-hydroxy-2-oxopropylphosphonate could have some precedence in the biodegradation of 2-aminoethylphosphonic acid (AEP) (Scheme 5, route B). It is first converted by a transaminase to phosphonoacetaldehyde (**22**) and then by phosphonatase to acetaldehyde and orthophosphate.[17] The latter enzyme was found to have an essential lysine in the active site. It forms a protonated Schiff base (imine) **23** with the substrate, having an electron sink at the β-carbon atom (relative to phosphorus), and accommodates the electron pair of the P–C bond when an enzyme nucleophile attacks phosphorus. Subsequent hydrolysis of the resultant acetaldehyde enamine **24** and the phosphoenzyme **25** gives acetaldehyde (**26**) and P_i. A similar mechanism could be operative in the cleavage of the P–C bond in (*R*)-1-hydroxy-2-oxopropylphosphonic acid, except that a hydroxyacetone enamine intermediate is involved, which is finally hydrolyzed to hydroxyacetone. Alternatively, a metal-ion-complexed enolate could be an equally good leaving group to facilitate fission of the P–C bond. More experiments are necessary to clarify this point. These two mechanisms are reminiscent of both the lysine and the Zn^{2+}-dependent fructose-1,6-bisphosphate aldolases of the glycolytic cycle. They cleave a C–C bond, with a protonated enamine and a metal-complexed enolate of dihydroxyacetone phosphate, respectively, serving as the leaving group.[18] Cleavage of the P–C bond in phosphonates without an electron sink at the β-carbon atom most likely involves radical intermediates.[19]



Scheme 5. Mechanisms for cleavage of P–C bonds.

Conclusion

We have shown that the isolation of alanine and a mixture of valine and methionine as *N*-acetyl derivatives by reversed-phase HPLC helped to unravel the biodegradation of fosfomycin in *R. huakuii* PMY1. Feeding with racemic (1*R**,2*R**)-1,2-dihydroxypropylphosphonic acid and hydroxyacetone, both ^{13}C-labeled at C-1, resulted in the formation of alanine and valine that were highly labeled at C-1. These findings are taken as evidence that (1*R*,2*R*)-1,2-dihydroxypropylphosphonic acid is converted into (*R*)-1-hydroxy-2-oxopropylphosphonic acid, which is the substrate for the enzyme cleaving the P–C bond. The hydroxyacetone formed is dehydrogenated to pyruvic acid via methylglyoxal. The former is used in part for the biosynthesis of amino acids. Therefore, our feeding experiments have also elucidated the biodegradation of hydroxyacetone by cells of *R. huakuii* PMY1.

Experimental Section

General: ^{1}H, ^{13}C (in part *J*-modulated, not for ^{13}C-labeled compounds), and ^{31}P NMR spectra were recorded from solutions in $CDCl_3$ or D_2O at 300 K on a Bruker DRX 400 (or Avance III 400) at 400.13, 100.61, and 161.98 MHz (or at 400.27, 100.64, and 162.03 MHz), respectively. Some ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 600 at 600.13 and 150.90 MHz, respectively. Chemical shifts were referenced to residual $CHCl_3$ ($\delta_H = 7.24$ ppm) or HDO ($\delta_H = 4.80$ ppm), $CDCl_3$ ($\delta_C = 77.00$ ppm), and external H_3PO_4 (85%, $\delta_P = 0.00$ ppm). IR spectra of liquid samples were recorded from films on a silicon disc[20] or between NaCl plates on a Perkin–Elmer 1600 FTIR spectrometer. IR spectra of crystalline compounds were recorded from samples on an attenuated total internal reflection (ATR) diamond on a Bruker Vertex 70 spectrometer. FID and HR-ESI mass spectra were recorded on a MAT 95S and a MAT 900S (Finnigan MAT), respectively. TLC was carried out on 0.25 mm thick Merck plates of silica gel 60 F_{254}. Spots were visualized by UV and/or by dipping the plate into a solution of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (23.0 g) and $Ce(SO_4)_2\cdot 4H_2O$ (1.0 g) in 10% aqueous H_2SO_4 (500 mL), followed by heating with a heat gun. Flash (column) chromatography was performed with Merck silica gel 60 (230–400 mesh). DMF and pyridine were dried by refluxing over powdered CaH_2, then distillation and storage over molecular sieves (4 Å). Dichloromethane and 1,2-dichloroethane were dried by passing through aluminum oxide 90 active neutral (0.063–0.200 mm, activity I) and stored over molecular sieves (3 and 4 Å, respectively). Hexane was dried by storage over molecular sieves (4 Å). Et_2O was refluxed over $LiAlH_4$, THF over potassium and distilled prior to use. Melting points were determined on a Reichert Thermovar instrument and are uncorrected.

Hydrolysis of cells and chromatography: Three aliquots of 2.0 mL of cell suspension (containing approximately 0.5–0.6 g of dry matter) were mixed with 6 M HCl (6 mL, containing 2.0% (w/v) phenol) and the mixture was heated at 95 °C for 8 h. After cooling, the hydrolysate was filtered through filter paper and concentrated in a rotary evaporator at 40 °C. The solution was diluted with water (3.0 mL) and then freeze-dried. The residue was redissolved in 3 M aqueous KOH (1.0 mL) and then Ac_2O (3.0 mL) was added. After 2 h, the solution was diluted with water to a volume of 40 mL and freeze-dried. The freeze-dried sample was redissolved in water (0.8 mL) and injected onto a Kromasil C18 column of dimensions 250×10 mm. A linear gradient of 0.02 M HCO_2H (eluent A) and MeOH/H_2O, 8:2 (v/v) (eluent B) was applied. The eluent composition was 10% B at the start of the gradient, 45% B at 12.0 min, 60% B at 13.0 min, and 100% B at 22.0 min. The flow rate was set at 5.0 mL min^{-1}. Acetylated amino acids were detected by means of a Merck L-3000 diode-array detector and fractions were collected with an Isco model Foxy fraction collector. Fractions containing *N*-acetylalanine were pooled and purified by reinjection. Isocratic elution was performed with a mixture of 0.02 M HCO_2H/MeOH, 4:1 (v/v). Pooled fractions containing *N*-acetylmethionine and *N*-acetylvaline were further purified on a Superspher C18 column of dimensions 250×4 mm. Isocratic elution was performed with a mixture of 0.01 M HCO_2H/MeOH, 9:1 (v/v) at a flow rate of 0.8 mL min^{-1}.

(±)-(2-Triisopropylsilyloxy)propanenitrile and (±)-2-triisopropylsilyloxy-[1-^{13}C$_1$]propanenitrile ((±)-8 and (±)-[1-^{13}C$_1$]8): Acetaldehyde (0.159 g, 0.20 mL, 3.60 mmol) was added to a stirred mixture of NaCN (0.147 g, 3.0 mmol, powdered), imidazole (0.450 g, 6.60 mmol), TIPSCl (0.617 g, 0.69 mL, 3.20 mmol), and dry DMF (5.0 mL) at 4 °C under argon. After stirring for 60 h at room temperature, water (20 mL) and Et_2O (40 mL) were added. The organic phase was removed and the aqueous phase was extracted with further Et_2O (20 mL). The combined organic layers were washed with HCl (1 M) and a saturated aqueous solution of $NaHCO_3$, dried ($MgSO_4$), and concentrated under reduced pressure. The residue was flash chromatographed (hexanes/CH_2Cl_2, 5:1, $R_f = 0.27$) to give nitrile (±)-**8** (0.550 g, 81%) as a colorless oil. ^{1}H NMR (400.13 MHz, $CDCl_3$): $\delta = 4.65$ (q, $J = 6.7$ Hz, 1H; CHCN), 1.58 (d, $J = 6.7$ Hz, 3H; CH_3), 1.22–1.04 ppm (m, 21H; *i*Pr); ^{13}C NMR (100.61 MHz, $CDCl_3$): $\delta = 120.7$, 58.1, 23.3, 17.8 and 17.7 (6C), 11.9 ppm (3C); IR (NaCl): $\tilde{\nu} = 2946$, 2868, 2230, 1464, 1375, 1132, 1065 cm^{-1}; elemental analysis calcd (%) for $C_{12}H_{25}NOSi$ (227.4): C 63.71, H 11.10; found: C 63.91, H 11.39.

Similarly, (±)-2-triisopropylsilyloxy-[1-^{13}C$_1$]propanenitrile ((±)-[1-^{13}C$_1$]**8**) (5.2 g, 85%) was prepared from acetaldehyde (1.54 g, 1.96 mL, 34.84 mmol, 1.3 equiv) and Na^{13}CN (1.34 g, 26.8 mmol, 99% ^{13}C). ^{1}H NMR (400.13 MHz, $CDCl_3$): $\delta = 4.63$ (dq, $J = 6.5$, 5.0 Hz, 1H; CHCN), 1.56 (dd, $J = 6.5$, 6.1 Hz; CH_3), 1.19–1.02 ppm (m, 21H; *i*Pr); ^{13}C NMR (100.61 MHz, $CDCl_3$): $\delta = 120.7$, 58.1 (d, $J = 61.2$ Hz; *C*H^{13}CN), 23.3, 17.8 and 17.7 (6C), 11.9 ppm (3C).

(1*R,2*R**)-(±)- and (1*S**,2*R**)-(±)-Diethyl 1-hydroxy-2-(triisopropylsilyloxy)-propylphosphonate ((±)-9 and (±)-10) and (1*R**,2*R**)-(±)-diethyl 1-hydroxy-2-(triisopropylsilyloxy)-[1-^{13}C$_1$]propylphosphonate ((±)-[1-^{13}C$_1$]9)**: (±)-2-(Triisopropylsilyloxy)propanal: Diisobutylaluminum hydride (DIBAH) (1 M solution in heptane, 6 mL, 6 mmol) was added dropwise to a stirred solution of (±)-2-(triisopropylsilyloxy)propanenitrile ((±)-**8**, 1.137 g, 5 mmol) in dry toluene (15 mL) at −78 °C under argon. The reaction mixture was then allowed to warm to 10 °C over a period of 16 h. Et_2O (30 mL), a saturated aqueous solution of NH_4Cl (30 mL), and H_2SO_4 (20 mL, 1.6 M) were added and the mixture was stirred vigorously for 0.5 h at ambient temperature. The organic phase was separated and the aqueous phase was extracted with Et_2O (3×30 mL). The combined organic phases were washed with water, dried ($MgSO_4$), and concentrated under reduced pressure. The crude product was dried by co-distillation with toluene and then maintained in vacuo (0.50 mbar) for 1 h to yield the crude aldehyde (1.049 g) as an oil, which was immediately used for the next step. Diethyl trimethylsilyl phosphite (1.37 mL, 1.265 g, 6.0 mmol) was added dropwise to a stirred solution of the above aldehyde (1.049 g) in dry toluene (20 mL) at −78 °C under argon. The reaction mixture was then allowed to warm to 10 °C over a period of 16 h. Volatile components were removed in a rotary evaporator at 0.5 mbar for 1 h to yield 2.56 g of the crude phosphonates as an oil, which was redissolved in dry ethanol (30 mL). Concentrated HCl (3 drops) was added and the mixture was stirred for 1 h (TLC: hexanes/acetone, 3:1, $R_f = 0.38$ for (±)-**9** and 0.30 for (±)-**10**). The solution was concentrated under reduced pressure and the residue was redissolved in water (10 mL). The aqueous layer was extracted with EtOAc (3×15 mL). The combined organic phases were dried ($MgSO_4$) and concentrated under reduced pressure. The residue ((±)-**9**/(±)-**10**, 7:1 by ^{31}P NMR) was purified by flash chromatography (hexanes/acetone, 5:1, $R_f = 0.28$ for (±)-**9** and 0.20 for (±)-**10**) to give (±)-**9** (1.172 g, 53% starting from nitrile) and (±)-**10** (0.155 g, 7%, containing 7% of (±)-**9**) as colorless oils.

(±)-9: ^{1}H NMR (400.27 MHz, $CDCl_3$): $\delta = 4.32$ (quintd, $J = 7.3$, 6.2 Hz, 1H; PCCH), 4.22–4.12 (m, 4H; 2 × OCH_2), 3.61 (td, $J = 6.2$, 4.9 Hz, 1H; PCH), 2.95 (dd, $J = 16.6$, 4.9 Hz, 1H; OH), 1.35 (d, $J = 6.2$ Hz, 3H; CH_3), 1.33 (t, $J = 7.1$ Hz; $OCCH_3$), 1.32 (t, $J = 7.1$ Hz, 3H; $OCCH_3$), 1.14–

1.04 ppm (m, 21H; 3 × *i*Pr); ^{13}C NMR (100.65 MHz, $CDCl_3$): δ = 72.9 (d, J = 162.9 Hz), 68.3 (d, J = 5.9 Hz), 62.6 (d, J = 6.6 Hz), 62.3 (d, J = 6.6 Hz), 21.5 (d, J = 5.9 Hz), 18.11 (3C), 18.02 (3C), 16.5 (d, J = 5.8 Hz, 2C), 12.7 ppm (3C); ^{31}P NMR (161.98 MHz, $CDCl_3$): δ = 22.2 ppm; IR (Si): $\tilde{\nu}$ = 3303, 2943, 2867, 1465, 1275, 1258, 1054, 1029, 971 cm^{-1}; elemental analysis calcd (%) for $C_{16}H_{37}O_5PSi$ (368.52): C 52.15, H 10.12; found: C 52.43, H 10.10.

(±)-10: ^{1}H NMR (400.27 MHz, $CDCl_3$): δ = 4.30 (qdd, J = 6.3, 5.8, 2.8 Hz, 1H; PCCH), 4.21–4.10 (m, 4H; 2 × OCH_2), 4.01 (dd, J = 10.8, 2.8 Hz, 1H; PCH), 2.35 (brs, 1H; OH), 1.32 (d, J = 6.3 Hz, 3H; CH_3), 1.31 (t, J = 7.1 Hz, 6H; 2 × $OCCH_3$), 1.10–1.00 ppm (m, 21H; 3 × *i*Pr); ^{13}C NMR (100.65 MHz, $CDCl_3$): δ = 72.9 (d, J = 167.9 Hz), 68.6 (d, J = 9.0 Hz), 62.7 (d, J = 7.0 Hz), 62.3 (d, J = 6.0 Hz), 18.4 (erroneously reported[10] to be a doublet at 17.7), 18.3 (3C), 18.2 (3C), 16.67 (d, J = 5.9 Hz), 16.64 (d, J = 5.9 Hz), 12.5 ppm (3C); ^{31}P NMR (161.98 MHz, $CDCl_3$): δ = 21.0 ppm; IR (Si): $\tilde{\nu}$ = 3306, 2943, 2867, 1465, 1242, 1258, 1141, 1096, 1054, 1028, 972 cm^{-1}; HRMS (ESI): *m*/*z*: calcd for $C_{16}H_{37}O_5PSiNa$ $[M+Na]^+$: 391.2046; found: 391.2052.

(±)-[1-^{13}C$_1$]9 and (±)-[1-^{13}C$_1$]10: In analogy to the preparation of the unlabeled species, (±)-2-(triisopropylsilyloxy)propane-[1-^{13}C$_1$]nitrile ((±)-[1-^{13}C$_1$]**8**), 1.60 g, 7.0 mmol) was converted into the crude aldehyde (1.37 g), which was immediately transformed into labeled hydroxyphosphonates (±)-[1-^{13}C$_1$]**9** (1.242 g, 48%, based on the starting nitrile) and (±)-[1-^{13}C$_1$]**10** (0.155 g, 6%, containing 16 mol% diethyl phosphite), both with 99% ^{13}C at C-1.

(±)-[1-^{13}C$_1$]**9**: ^{1}H NMR (400.13 MHz, $CDCl_3$): δ = 4.30 (m, 1H; CHCP), 4.15 (sext, J = 7.0 Hz, 4H; 2 × OCH_2), 3.59 (td, J = 142.6, 6.0 Hz, 1H; ^{13}CHP), 2.78 (brs, 1H; OH), 1.35 (t, J = 6.0 Hz, 3H), 1.31 (t, J = 7.0 Hz, 3H; $OCCH_3$), 1.30 (t, J = 7.0 Hz, 3H; $OCCH_3$), 1.18–1.00 ppm (m, 21H; 3 × *i*Pr); ^{13}C NMR (100.61 MHz, $CDCl_3$): δ = 72.8 (d, J = 162.3 Hz; ^{13}CHP), 68.3 (dd, J = 37.9, 5.8 Hz; *C*^{13}CP), 62.6 (d, J = 6.8 Hz; OCH_2), 62.2 (d, J = 6.8 Hz; OCH_2), 21.4 (dd, J = 5.8, 1.9 Hz; CH_3), 18.09 and 18.00 (2s, 6C), 16.4 (d, J = 5.8 Hz, 2C), 12.7 ppm (3C); IR (Si): $\tilde{\nu}$ = 3303, 2943, 2867, 1465, 1275, 1258, 1054, 1029, 971 cm^{-1}.

(±)-[1-^{13}C$_1$]**10**: ^{1}H NMR (400.13 MHz, $CDCl_3$): δ = 4.30 (m, 1H; CHCP), 4.21–4.10 (m, 4H; 2 × OCH_2), 4.01 (ddd, J = 140.5, 10.5, 3.0 Hz, 1H; ^{13}CHP), 2.55 (brs, 1H; OH), 1.32 (d, J = 7.0 Hz, 3H; CH_3), 1.31 (t, J = 7.0 Hz, 6H; 2 × $OCCH_3$), 1.12–0.98 ppm (m, 21H; 3 × *i*Pr); ^{13}C NMR (100.61 MHz, $CDCl_3$): δ = 72.9 (d, J = 161.7 Hz; ^{13}CHP), 68.6 (dd, J = 37.9, 9.3 Hz; *C*CP), 62.7 (d, J = 6.9 Hz), 62.3 (d, J = 6.1 Hz), 18.1, 18.02, and 17.98 (2s, 6C), 16.42 (d, J = 5.4 Hz), 16.40 (d, J = 5.4 Hz), 12.3 ppm (3C); ^{31}P NMR (161.98 MHz, $CDCl_3$): δ = 22.3 ppm (d, J = 161.7 Hz; ^{13}CHP); MS (FID): *m*/*z* (%): 369 $[M^+]$ (1), 370 (100).

(1*R,2*R**)-(±)-Diethyl 1,2-dihydroxypropylphosphonate and (1*R**,2*R**)-(±)-diethyl 1,2-dihydroxy-[1-^{13}C$_1$]propylphosphonate ((±)-11 and (±)-[1-^{13}C$_1$]11)**: A solution of TIPS-protected diol (±)-**9** (0.654 g, 1.77 mmol) in dry CH_3CN (15 mL), water (7 drops), and HF (40%, 10 drops) was left at room temperature in a small plastic bottle until the starting material had been consumed (22 h) and then concentrated under reduced pressure in a round-bottomed glass flask. The residue was flash-chromatographed (EtOAc/MeOH, 20:1, R_f = 0.14) to give diol (±)-**11** (0.368 g, 98%) as a viscous oil. ^{1}H NMR (400.27 MHz, $CDCl_3$): δ = 4.27–4.08 (m, 5H; 2 × OCH_2, PCCH), 3.65 (brt, J = 6.5 Hz, 1H; CHP), 3.35–3.00 (two broad overlapping s, 2H; OH), 1.34 (t, J = 7.0 Hz, 3H; CH_3), 1.33 (t, J = 7.0 Hz, 3H; CH_3), 1.29 ppm (dd, J = 6.2, 1.5 Hz, 3H; CH_3); ^{13}C NMR (100.65 MHz, $CDCl_3$): δ = 71.4 (d, J = 159.1 Hz), 66.4, 63.46 (d, J = 7.3 Hz), 62.49 (d, J = 7.3 Hz), 19.0 (d, J = 11.7 Hz), 16.47 (d, J = 5.1 Hz), 16.44 ppm (d, J = 5.1 Hz); ^{31}P NMR (162.03 MHz, $CDCl_3$): δ = 23.3 ppm; IR (Si): $\tilde{\nu}$ = 3317, 2979, 2940, 2867, 1462, 1391, 1275, 1053, 1029, 971 cm^{-1}; elemental analysis calcd (%) for $C_7H_{17}O_5P$ (212.18): C 39.62, H 8.08; found: C 39.47, H 7.83.

(±)-[1-^{13}C$_1$]11: Similarly, TIPS-protected diol (±)-[1-^{13}C$_1$]**9** (1.12 g, 3.03 mmol) was converted into diol (±)-[1-^{13}C$_1$]**11** (0.592 g, 92%). ^{1}H NMR (400.13 MHz, $CDCl_3$): δ = 4.16 (m, 5H; 2 × OCH_2, PCCH), 3.90 (ddd, J = 9.0, 2.5, 2.0 Hz, 1H; OH), 3.64 (tdd, J = 142.0, 9.0, 3.0 Hz, 1H; ^{13}CHP), 3.54 (d, J = 4.5 Hz, 1H; OH), 1.32 (t, J = 7.0 Hz, 3H; CH_3), 1.31 (t, J = 7.0 Hz, 3H; CH_3), 1.27 ppm (ddd, J = 5.0, 4.5, 1.5 Hz, 3H; CH_3); ^{13}C NMR (100.61 MHz, $CDCl_3$): δ = 71.8 (d, J = 159.4 Hz; ^{13}CHP), 66.5 (dd, J = 36.9, 2.9 Hz; CHCP), 63.3 (d, J = 6.8 Hz), 62.6 (d, J = 6.8 Hz), 19.1 (d, J = 10.7 Hz; CH_3), 16.42 (d, J = 4.9 Hz; CH_3), 16.39 ppm (d, J = 5.8 Hz; CH_3); ^{31}P NMR (161.98 MHz, $CDCl_3$): δ = 24.8 ppm (d, J = 158.5 Hz; ^{13}CHP); MS (FID): *m*/*z* (%): 213 $[M^+]$ (2.5), 214 (100).

(1*R,2*R**)-(±)-1,2-Dihydroxypropylphosphonic acid and (1*R**,2*R**)-(±)-1,2-dihydroxy-[1-^{13}C$_1$]propylphosphonic acid ((±)-3 and (±)-[1-^{13}C$_1$]3)**: **(±)-3**: A solution of (1*R**,2*R**)-(±)-1,2-dihydroxypropylphosphonate (±)-**11** (0.368 g, 1.73 mmol), allylTMS (0.985 g, 1.38 mL, 8.65 mmol, 5 equiv), and bromotrimethylsilane (3.337 g, 2.88 mL, 21.8 mmol) in dry 1,2-$C_2H_4Cl_2$ (8.0 mL) was kept under argon for 16 h at room temperature. Volatile components were then removed in vacuo (0.50 mbar, ambient to 40°C) and the residue was dissolved in a 1:1 mixture of EtOH/H_2O (10 mL). The solvent was removed under reduced pressure and the residue was taken up in water (10 mL) and concentrated NH_3 (1.0 mL) and then lyophilized to leave a gummy residue of the ammonium salt of (±)-**3** (0.30 g, containing an unknown amount of ammonia and water; the yield was assumed to be 95%). The spectroscopic data (^{1}H, ^{13}C, and ^{31}P) were identical to those of the ammonium salt of the (1*R*,2*R*)-dihydroxypropylphosphonic acid derived from fosfomycin. If desired, the ammonium salt can be purified by the method used for the conversion of the cyclohexylammonium salt of 1,2-dihydroxypropylphosphonic acid to the ammonium salt.[10]

(±)-[1-^{13}C$_1$]3·(NH$_3$)$_x$: The labeled 1,2-dihydroxypropylphosphonate (±)-[1-^{13}C$_1$]**11** (0.337 g, 1.59 mmol) was converted into the ammonium salt of (±)-[1-^{13}C$_1$]**3** following the procedure used for the unlabeled species. ^{1}H NMR (400.13 MHz, D_2O): δ = 4.05 (m, 1H; CHCP), 3.50 (ddd, J = 138.0, 9.5, 4.5 Hz, 1H; ^{13}CHP), 1.31 ppm (t, J = 5.5 Hz, 3H; CH_3); ^{13}C NMR (100.61 MHz, D_2O): δ = 73.3 (d, J = 141.5 Hz; ^{13}CHP), 68.3 (d, J = 39.8 Hz; *C*CP), 18.8 ppm (d, J = 8.4 Hz; CH_3); ^{31}P NMR (161.97 MHz, D_2O): δ = 17.0 ppm (d, J = 141.7 Hz).

2-Methyl-2-propenyl 4-nitrobenzoate and 2-methyl-2-[1-^{13}C$_1$]propenyl 4-nitrobenzoate (17 and [1-^{13}C$_1$]17): A solution of isopropenylmagnesium bromide (0.5 M in THF, 20 mL, 10.0 mmol) was added to a suspension of paraformaldehyde (0.150 g, 5.0 mmol) in dry Et_2O (2 mL) at −20°C under argon. The mixture was stirred overnight at room temperature. After careful addition of H_2O (10 mL) and dissolution of the precipitate with dilute H_2SO_4, the organic phase was separated and the aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL). The combined organic solutions were dried (Na_2SO_4) and filtered (the Na_2SO_4 was washed with dry CH_2Cl_2), and most of the CH_2Cl_2 was distilled off at atmospheric pressure over a short column. The resulting THF solution of the alcohol was stirred at room temperature under argon for 30 min with molecular sieves (3.5 g, 4 Å). The mixture was then cooled to 0°C, whereupon 4-nitrobenzoyl chloride (1.45 g, 7.81 mmol), dry pyridine (2.6 mL), and DMAP (52 mg) were successively added. After stirring overnight at room temperature, the solution was decanted from the molecular sieves (which were rinsed with CH_2Cl_2), and the combined solutions were treated with H_2O (30 mL) and concentrated HCl (about 4.5 mL, to dissolve the precipitate). The organic layer was separated and the aqueous phase was extracted twice with CH_2Cl_2. The combined organic phases were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by flash chromatography (hexane/CH_2Cl_2, 1:1, R_f = 0.50 for hexane/EtOAc, 10:1; 0.55 for hexane/CH_2Cl_2, 1:1) to yield 4-nitrobenzoate **17** (0.686 g, 62%) as yellowish crystals; m.p. 68–70°C (hexane/a few drops of 1,2-$C_2H_4Cl_2$; lit.:[21] no data). (400.13 MHz, $CDCl_3$): δ = 8.25 (AA′BB′ system, 4H; H_{ar}), 5.07 (brs, 1H; =CH), 5.00 (brs, 1H; =CH), 4.77 (brs, 2H; OCH_2), 1.83 ppm (brs, 3H; CH_3); (100.61 MHz, $CDCl_3$): δ = 164.33, 150.59, 139.31, 135.56, 130.72 (2C), 123.56 (2C), 113.76, 69.03, 19.55 ppm; IR (ATR): $\tilde{\nu}$ = 3117, 1710, 1520, 1268, 1119 cm^{-1}; elemental analysis calcd (%) for $C_{11}H_{11}NO_4$ (221.2): C 59.73, H 5.01, N 6.33; found: C 59.85, H 4.79, N 6.28.

Similarly, ^{13}C-labeled paraformaldehyde (0.162 g, 5.23 mmol) gave [1-^{13}C$_1$]**17** (0.789 g, 68%), 99% C-13 labeled, m.p. 62–63°C (hexane).

[1-^{13}C$_1$]17: ^{1}H NMR (400.13 MHz, $CDCl_3$): δ = 8.24 (AA′BB′ system, 4H; H_{ar}), 5.06 (d, J = 6.8 Hz, 1H; =CH), 4.99 (d, J = 12.13 Hz, 1H; =CH), 4.76 (d, J = 147.8 Hz, 2H; O^{13}CH$_2$), 1.82 ppm (d, J = 4.29 Hz, 3H; Me); ^{13}C NMR (100.61 MHz, $CDCl_3$): δ = 164.33 (d, J = 2.3 Hz; CO), 150.59 (C_{ar}), 139.30 (d, J = 46.0 Hz; C=), 135.56 (C_{ar}), 130.73 (2 × CH_{ar}), 123.56

(2 × CH_{ar}), 113.75 (d, J=3.1 Hz; CH_2=), 69.03 ($^{13}CH_2$), 19.55 ppm (d, J=5.4 Hz; Me).

2-Oxo-propyl 4-nitrobenzoate and 2-oxo-[1-$^{13}C_1$]propyl 4-nitrobenzoate (18 and [1-$^{13}C_1$]18): 4-Nitrobenzoate **17** (0.665 g, 3.0 mmol) was dissolved in a mixture of dry CH_2Cl_2 (15 mL) and dry MeOH (15 mL), and the solution was cooled to −78 °C and subjected to ozonolysis. Thereafter, triphenylphosphane (3.1 mmol) was added to the cold solution, which was then stirred overnight and allowed to warm to room temperature. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography (hexane/EtOAc, 5:1, then 1:1; R_f= 0.61 for 1:1) to yield **18** (0.647 g, 96%) as yellowish crystals; m.p. 96–98 °C (hexane/1,2-$C_2H_4Cl_2$).

19: ^{1}H NMR (400.13 MHz, $CDCl_3$): δ=8.27 (AA′BB′ system, 4H; H_{ar}), 4.93 (s, 2H; OCH_2), 2.23 ppm (s, 3H; CH_3); ^{13}C NMR (100.61 MHz, $CDCl_3$): δ=200.23 (CO), 163.98 (CO_2), 150.81 (C_{ar}), 134.59 (C_{ar}), 131.03 ($2CH_{ar}$), 123.62 ($2CH_{ar}$), 69.14 (OCH_2), 26.07 ppm (CH_3); IR (ATR): $\tilde{\nu}$= 1743, 1720, 1617, 1347, 1272, 1103 cm^{-1}; elemental analysis calcd (%) for $C_{10}H_9NO_5$ (223.2): C 53.82, H 4.06, N 6.28; found: C 53.72, H 3.90, N 6.20.

Similarly, 4-nitrobenzoate [1-$^{13}C_1$]**17** (2.39 g, 10.80 mmol) was converted into [1-$^{13}C_1$]**18** (2.206 g, 91%); m.p. 95 °C (hexane/CH_2Cl_2), 98% ^{13}C at C-1. ^{1}H NMR (400.13 MHz, $CDCl_3$): δ=8.25 (AA′BB′ system, 4H; H_{ar}), 4.93 (d, J=147.3 Hz, 2H; O$^{13}CH_2$), 2.22 ppm (d, J=2.3 Hz, 3H; Me); ^{13}C NMR (100.61 MHz, $CDCl_3$): δ=200.24 (d, J=40.6 Hz; CO), 163.96 (d, J=2.3 Hz; CO_2), 150.78 (C_{ar}), 135.00 (d, J=1.3 Hz; C_{ar}), 131.01 (2 × CH_{ar}), 123.60 (2 × CH_{ar}), 69.12 (O$^{13}CH_2$), 26.04 ppm (d, J=18.4 Hz; Me).

Hydroxyacetone and 1-hydroxy-[1-$^{13}C_1$]acetone (5 and [1-$^{13}C_1$]5): Ester **18** (2.01 g, 9.0 mmol) was dissolved in dry CH_2Cl_2 (28 mL) and the solution was cooled to −20 °C under argon. Then, a solution of NaOMe in MeOH, which was prepared by dissolving Na (70 mg) in dry MeOH (2.8 mL), was added. After stirring for exactly 20 min at −20 °C, small pieces of dry ice (about 0.4 g) were added to neutralize the solution, which was immediately applied to a flash chromatography column (about 55 g of silica gel, 2×37 cm, CH_2Cl_2 as eluent). Fractions containing the product (TLC: hexane/EtOAc, 1:1, R_f=0.26) were collected (150–200 mL) and the solvent was evaporated under reduced pressure (rotary evaporator, flask not in the water bath) until only a small amount was left. The residue was transferred to a small flask using Et_2O and bulb-to-bulb distilled (90–100 mmHg/120 °C final bath temperature) to yield hydroxyacetone (**5**) (0.347 g, 52%) as a liquid containing a small amount of impurities. The ^{1}H and ^{13}C NMR spectra were identical to those of an authentic sample.

Analogously, labeled ester [1-$^{13}C_1$]**17** (2.18 g, 9.72 mmol) was converted into labeled hydroxyacetone [1-$^{13}C_1$]**5** (0.430 g, 59%), 97% C-1 labeled. ^{1}H NMR (400.13 MHz, $CDCl_3$): δ=4.19 (d, J=131.4 Hz; O$^{13}CH_2$), 3.07 (br s, 1H; OH), 2.11 ppm (d, J=2.3 Hz, 3H; Me); ^{13}C NMR (100.61 MHz, $CDCl_3$): δ=207.1 (d, J=37.6 Hz; CO), 68.6 (O$^{13}CH_2$), 25.2 ppm (d, J=16.1 Hz; CH_3).

***N*-Acetylalanine**: ^{1}H NMR (400.13 MHz, D_2O): δ=4.28 (q, J=7.5 Hz, 1H; $CHCO_2$), 1.96 (s, 3H; CH_3CO), 1.35 ppm (d, J=7.5 Hz, 3H; CH_3; J(^{13}C,H)=129.5 Hz); ^{13}C NMR (100.61 MHz, D_2O): δ=177.2, 174.4, 49.1, 21.9, 16.4 ppm; HCO_2H: 8.2 ppm.

***N*-Acetylvaline**: ^{1}H NMR (400.13 MHz, D_2O): δ=4.27 (d, J=6.0 Hz, 1H; $CHCO_2$), 2.23 (≈oct, J=6.5 Hz, 1H; CH), 2.10 (s, 3H; CH_3), 1.01 (d, J=6.5 Hz, 3H; CH_3), 1.00 ppm (d, J=6.5 Hz, 3H; CH_3); ^{13}C NMR (100.61 MHz, D_2O): δ=176.1, 174.9, 59.0, 30.1, 21.9, 18.6, 17.5 ppm.

***N*-Acetylmethionine**: ^{1}H NMR (600.13 MHz, D_2O): δ=4.55 (dd, J=9.6, 4.5 Hz, 1H; $CHCO_2$), 2.71–2.58 (m, 2H; SCH_2), 2.27–2.19 (m, 1H; CH), 2.14 (s; SCH_3 or CH_3CO; J(^{13}C,H)=136.9 Hz), 2.09–2.03 (m, 1H; CH), 2.08 ppm (s; SCH_3 or CH_3CO); ^{13}C NMR (100.61 MHz, D_2O): δ=175.5, 174.4, 51.7, 29.4, 21.6, 14.1 ppm.

Microorganism and culture conditions: Cells of *Rhizobium huakuii* PMY1[9] were grown in batch cultures at 30 °C on an orbital shaker at 100 rpm in minimal salt medium (pH 7.0) of the following composition (per liter): KCl, 0.20 g; $MgSO_4 \cdot 7H_2O$, 0.20 g; $CaCl_2 \cdot 2H_2O$, 1.0 mg; NH_4Cl, 1.0 g; ferric ammonium citrate, 1.0 mg; phosphate-free yeast extract (0.05 g),[9] and 1 mL each of trace element solution and vitamin solution.[9] Either filter-sterilized (0.22 μm) fosfomycin (5 mM) or the respective organophosphonate under test as a potential pathway intermediate was added as the sole carbon and phosphorus source. Microbial growth was measured by the increase in absorbance at 650 nm using an ATI-Unicam PU 8625 UV/Vis spectrophotometer, while phosphate release into the culture supernatant was monitored according to the method of Fiske and SubbaRow.[22]

Feeding of (±)-[1-$^{13}C_1$]3: Culture (500 mL, in a 2 L flask) with (±)-[1-$^{13}C_1$]**3** (7.5 mM) was shaken for 11 days; the release of P_i amounted to 2.2 mmol. The cells were harvested by centrifugation, then sterilized and hydrolyzed.

Enzyme assays: Cell extracts of fosfomycin-grown cells were prepared by sonication as previously described.[9] Both acetol dehydrogenase and methylglyoxal dehydrogenase were assayed according to the method of Taylor et al.[15] by measuring the production of NADH at 340 nm. The assay mixture (1 mL) consisted of glycine-NaOH buffer pH 10 (90 μmol), NAD (0.5 μmol), and either acetol (2 μmol) or methylglyoxal (2 μmol) as substrate. The reaction was initiated by the addition of 20 μL of enzyme solution (containing 1–1.5 mg mL^{-1} of cell-extract protein) and the mixture was incubated for 30 min at 20 °C. Acetol monooxygenase was assayed by the method of Hartmans and de Bont[16] through measurement of NADPH consumption at 340 nm. The reaction mixture (1 mL) consisted of potassium phosphate buffer (0.3 mmol) pH 8.0, NADPH (0.8 μmol), and acetol (8 μmol). The reaction was initiated as described above. One enzyme unit is defined as the amount leading to either the consumption of 1 nmol of substrate or the formation of 1 nmol of product per min at 20 °C. For all enzyme assays, controls containing no crude cell extract or substrate were prepared to evaluate the degree of background activity. Protein concentration was determined by the method of Bradford using bovine serum albumin as standard.[23]

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[1] For a review, see: H. Seto, T. Kuzuyama, *Nat. Prod. Rep.* **1999**, *16*, 589–596.
[2] F. M. Kahan, J. S. Kahan, P. J. Cassidy, H. Kropp, *Ann. N. Y. Acad. Sci.* **1974**, *235*, 364–386.
[3] For a review, see: W. W. Metcalf, W. A. van der Donk, *Ann. Rev. Biochem.* **2009**, *78*, 65–94.
[4] a) H. M. Seidel, S. Freeman, C. H. Schwalbe, J. R. Knowles, *J. Am. Chem. Soc.* **1990**, *112*, 8149–8155; b) M. S. McQueney, S.-L. Lee, W. H. Swartz, H. L. Ammon, P. S. Mariano, D. Dunaway-Mariano, *J. Org. Chem.* **1991**, *56*, 7121–7130.
[5] F. Hammerschmidt, *J. Chem. Soc. Perkin Trans. 1* **1991**, 1993–1996.
[6] R. D. Woodyer, G. Li, H. Zhao, W. A. van der Donk, *Chem. Commun.* **2007**, 359–361.
[7] a) L. J. Higgins, F. Yan, P. Liu, H.-w. Liu, C. L. Drennan, *Nature* **2005**, *437*, 838–844; b) K. McLuskey, S. Cameron, F. Hammerschmidt, W. N. Hunter, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 14221–14226.
[8] a) A. K. White, W. W. Metcalf, *Ann. Rev. Microbiol.* **2007**, *61*, 379–400; b) J. P. Quinn, A. N. Kulakova, N. A. Cooley, J. W. McGrath, *Environ. Microbiol.* **2007**, *9*, 2392–2400; c) N. G. Ternan, J. W. McGrath, G. McMullan, J. P. Quinn, *World J. Microbiol. Biotechnol.* **1998**, *14*, 635–647.
[9] J. W. McGrath, F. Hammerschmidt, J. P. Quinn, *Appl. Environ. Microbiol.* **1998**, *64*, 356–358.
[10] J. W. McGrath, F. Hammerschmidt, W. Preusser, J. P. Quinn, A. Schweifer, *Org. Biomol. Chem.* **2009**, *7*, 1944–1953.
[11] E. Y. Lau, Z. E. Newby, T. C. Bruice, *J. Am. Chem. Soc.* **2001**, *123*, 3350–3357.

[12] K. L. Fillgrove, S. Pakhomova, M. E. Newcomer, R. N. Armstrong, *J. Am. Chem. Soc.* **2003**, *125*, 15730–15731.
[13] G. Cainelli, M. Panunzio, E. Bandini, G. Martellim, G. Spunta, *Tetrahedron* **1996**, *52*, 1685–1698.
[14] A. L. Lehninger, *Biochemistry*, 2nd. ed., Worth Publishers, New York, **1978**.
[15] D. G. Taylor, P. W. Trudgill, R. E. Cripps, P. R. Harris, *J. Gen. Microbiol.* **1980**, *118*, 159–170.
[16] S. Hartmans, J. A. M. de Bont, *FEMS Microbiol. Lett.* **1986**, *36*, 155–158.
[17] a) A.-M. Lacoste, C. Dumora, A. Cassaigne, *Life Sci. Adv.: Biochem.* **1989**, *8*, 97–111; b) S.-L. Lee, T. W. Hepburn, W. H. Swartz, H. L. Ammon, P. S. Mariano, D. Dunaway-Mariano, *J. Am. Chem. Soc.* **1992**, *114*, 7346–7354.
[18] K. Faber, *Biotransformations in Organic Chemistry*, 5th ed. Springer, Berlin, **2004**, p. 273.
[19] J. W. Frost, S. Loo, M. L. Cordeiro, D. Li, *J. Am. Chem. Soc.* **1987**, *109*, 2166–2171.
[20] W. Mikenda, *Vib. Spectrosc.* **1992**, *3*, 327–330.
[21] C. R. Schmid, *Tetrahedron Lett.* **1992**, *33*, 757–760.
[22] C. H. Fiske, Y. Subbarow, *J. Biol. Chem.* **1925**, *66*, 375–400.
[23] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248–254.

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