133.33, 134.47, 137.92, 163.27; IR ($CDCl_3$): $\tilde{\nu}$[cm^{-1}] = 2120 (N=C=S); FAB$^+$-MS (high resolution): calcd. 246.1065 (M^+); found 246.1063. Epitope densities at 276 nm (27 for KLH; 14 for BSA) and protein concentrations were determined as described above.

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[10] 7: ^{1}H NMR (300 MHz, CD_3OD): δ = 1.5–1.7 (m, 4H), 2.3 (t, ^3J(H,H) = 7 Hz, 2H), 3.1 (t, ^3J(H,H) = 7 Hz, 2H), 3.5 (s, 2H), 3.6 (m, 1H), 3.9–4.0 (m, 1H), 4.1–4.2 (m, 1H), 4.3–4.4 (m, 2H), 4.8 (d, ^3J(H,H) = 2 Hz, 1H), 7.6 (t, ^3J(H,H) = 7 Hz, 1H), 7.7 (d, ^3J(H,H) = 7 Hz, 1H), 8.1–8.2 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD): δ = 24.1, 30.5, 36.7, 40.1, 41.5, 64.3, 70.5, 78.5, 102.1, 122.5, 124.6, 130.5, 136.4, 141.7, 149.7, 154.1, 158.9, 180.5; FAB$^+$-MS (high resolution): calcd. 381.0814 (MH^+); found 381.0819. E.D. (262 nm) = 25 (KLH) and 28 (BSA).

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[12] Substrate 1 was synthesized by coupling 1-acetyl-5-bromoindole with 2-chloropyran under basic conditions followed by deacylation and purification by RP-HPLC. Cell supernatants or ascites fluid were diluted with an equal volume of MES buffer (pH 5.5) containing 2 mM substrate and 20% (v/v) DMF. After incubation at 37 °C for 12 h, indigo color was observable.

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[14] Assays were initiated by adding a stock solution of substrate 1 in DMF to the antibody in the assay buffer at 37 °C (final antibody concentration 6.67 µM; 20% (v/v) DMF). Hydrolysis was monitored with a Microsorb C18 reversed-phase column (4.6 mm × 25 cm) using a 10–60% acetonitrile/water gradient and an internal standard (3-nitrobenzonitrile). Reaction velocities were measured at less than 10% conversion.

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[16] Substrates 2 and 4 were synthesized by condensing 3,4-dihydro-2*H*-pyran with *m*-nitrobenzyl alcohol and *m*-nitrophenol, respectively. Hydrogenation of the former product with palladium on carbon, followed by reaction with thiophosgene in the presence of Hünig's base produced the isothiocyanate. Reaction of the isothiocyanate with ethyl amine afforded substrate 2. Assay conditions: 6.67 µM antibody, 250 µM substrate, 10% (v/v) CH_3CN in reaction buffer, 22 °C. Product formation was monitored by RP-HPLC as described with 2,4-dimethoxybenzamide as an internal standard.

[17] Substrate 3 was synthesized by condensing α-D-glucose pentaacetate with *m*-nitrophenol in the presence of a catalytic amount of $TiCl_4$ in CH_2Cl_2 at room temperature, followed by deacylation in K_2CO_3/CH_3OH. The ratio of anomers was 3:1 (α:β). Assay conditions: 6.67 µM antibody, 2.5 mM substrate, 10% (v/v) DMF. Product formation was monitored with a Microsorb phenyl column (4.6 mm × 15 cm).

[18] Enzyme-linked immunosorbent assays (ELISAs) with substrate–BSA conjugates indicated that 71 and 63% of the antibodies against haptens 6 and 7, respectively were capable of binding their substrates.

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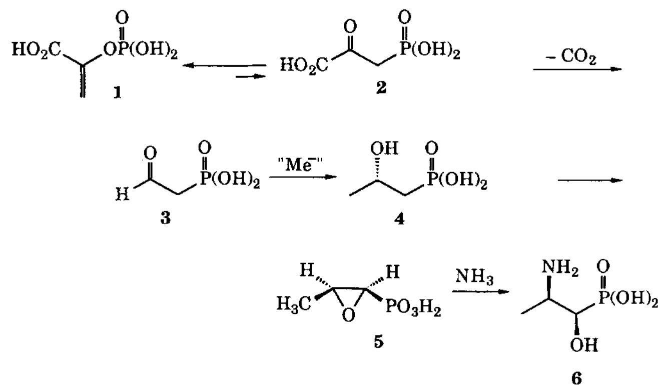
Incorporation of L-[Methyl-$^2H_3$]methionine and 2-[Hydroxy-^{18}O]hydroxyethylphosphonic Acid into Fosfomycin in *Streptomyces fradiae*—An Unusual Methyl Transfer

Friedrich Hammerschmidt*

Fosfomycin (5) is a clinically used antibiotic which belongs to the group of natural products with a P–C bond.[1] Its biosynthesis has two most interesting steps: the extension of a P–C_2 unit by a C_1 unit and the formation of an oxirane. The biogenesis of fosfomycin begins with the isomerization[2] of phosphoenolpyruvate (1) to give phosphonopyruvic acid (2), which presumably decarboxylates to afford phosphonoacetaldehyde (3),[3] which in turn is methylated to provide (*S*)-2-hydroxypropylphosphonic acid (4).[3–5] The latter is converted into fosfomycin (5); the oxirane oxygen originates from the hydroxyl group.[4] This paper provides further experimental evidence for the assumption previously made[6] that in the transformation of 3 into 4 a methyl group, formally CH_3^-, adds to the carbonyl carbon. There is no biosynthetic precedent for this step.[7]



Both the methyl group[3] of [methyl-^{14}C]methionine and the 2-hydroxy[2,2-$^2H_2$]ethylphosphonic acid (cf. 9)[6,8] are incorporated into fosfomycin. The latter is incorporated with retention of one of the deuteriums via a postulated aldehyde intermediate,[5,6,8] which is indeed plausible in the general context of biosynthesis of natural products with a P–C bond. Umpolung of the C=O functionality via a thiamine pyrophosphate adduct must be ruled out. Hence, a feeding experiment with L-[methyl-$^2H_3$]methionine (300 mg L^{-1}) with *Streptomyces fradiae* was undertaken to determine whether the methyl group was transferred intact, that is, without replacement of any of the deuteriums by protium. The fosfomycin produced (8 mg L^{-1}) was transformed into the labeled 2-amino-1-hydroxypropylphosphonic acid (6),[9] which was easily isolated and analyzed by NMR spectroscopy. In the ^{1}H NMR spectrum (400.1 MHz, D_2O) the pattern of signals and their integration show that 6 consists of 19% unlabeled and 81% trideuteriated compound. This can be deduced from the sharp doublet (^3J(H,H) = 7.0 Hz) at δ = 1.54 for 3-H and from the triplet (^3J(P,H) = ^3J(H,H) = 5.0 Hz) at δ = 3.72 for 2-H. In the ^{13}C{^{1}H} NMR spectrum (100.6 MHz, D_2O, sodium 4,4-dimethyl-4-silapentanesulfonate (DSS)) this result is supported by satellite doublets (isotope-induced β and γ shifts)[10] upfield for C-1 (δ = 72.917, ^1J(P,C) = 155.0 Hz, $\Delta\delta$ = 0.04) and C-2 (δ = 53.314, ^2J(P,C) = 4.5 Hz, $\Delta\delta$ = 0.16) for the labeled compound. The presence of a mono- or a dideuteriated species must be ruled out, as in the ^{1}H NMR spectrum these would give rise to additional doublets for 3-H, which would be considerably broadened through coupling with either one or two deuterium atoms. Additional satellite doublets for C-2 in the ^{13}C NMR spectrum would also be expected. Thus, the methyl group is transferred intact.

In order to determine whether phosphonoacetaldehyde (3) itself or another, as yet unknown, compound serves as the acceptor for the methyl group, 2-[hydroxy-^{18}O]hydroxyethylphosphonic acid (9) was synthesized from acetal 7 and added to the growth medium (470 mg L^{-1}). 9 is dehydrogenated by a dehydrogenase to give the aldehyde and is then taken up into the biosynthetic pathway. The ease with which oxygen exchanges in aldehydes appears to render [formyl-^{18}O]phosphonoacetaldehyde (cf. 3) an unsuitable substrate. The half-life for the exchange of oxygen

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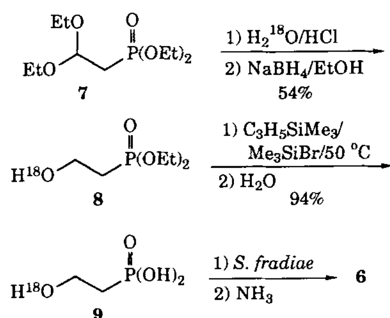
in acetaldehyde is approximately 1 min in neutral aqueous solution.[11] In the cell the [formyl-^{18}O]phosphonoacetaldehyde generated in situ should be methylated quickly before ^{18}O is completely exchanged. For the synthesis of **9**, diethyl 2,2-diethoxyethylphosphonate (**7**)[12] was hydrolyzed in $H_2{}^{18}O$ (95% ^{18}O) with HCl formed by addition of chlorotrimethylsilane. After three days the mixture (molar ratio of aldehyde **3**/acetal **7** = 1:1 after two days; ^{1}H NMR) was reduced with sodium borohydride in anhydrous ethanol. The ^{18}O-labeled hydroxyphosphonate **8** corresponded to the unlabeled compound (thin-layer chromatography (TLC), boiling point, ^{1}H NMR and IR spectra) which was obtained by the same procedure, except that H_2O was employed for the hydrolysis of **7** instead of $H_2{}^{18}O$. The electron impact (EI) mass spectrum of **7** showed that ^{16}O of the P=O bond had not been exchanged for ^{18}O.



According to the field desorption (FD) mass spectrum, the extent of labeling was at least 92%. The $^{13}C\{^1H\}$ NMR spectrum (100.6 Hz, $CDCl_3$) of the labeled compound **8** shows the C-2 doublet to be upfield ($\delta = 56.417$, $^2J(C,P) = 4.6$ Hz, $\Delta\delta = 0.018$). **8** was treated with bromotrimethylsilane/allytrimethylsilane[9, 13] and finally hydrolyzed to give the free phosphonic acid **9**, which was added to the growth medium (470 mg L^{-1}). The labeled fosfomycin that formed (10 mg L^{-1}) was once again transformed into 2-amino-1-hydroxypropylphosphonic acid and isolated. The ^{1}H NMR spectrum (400.1 MHz, D_2O) of this material is identical to that of the unlabeled compound **6**. The $^{13}C\{^1H\}$ spectrum obtained after the addition of NaOD (30%) in D_2O[9] showed that adjacent to the doublet ($\delta = 78.885$, ^1J(P,C = 144.7 Hz) for C-1 is a satellite doublet with the same intensity as for ^{18}O-labeled **6** which is shifted upfield by $\Delta\delta = 0.025$. This corresponds to 50% labeling. It is evident, therefore, that the labeled phosphonoacetaldehyde has a short lifetime in the cell. According to these two experiments the methyl group is probably transferred formally as CH_3^- which, however, rules out L-methionine and (*S*)-adenosylmethionine (which deliver the methyl group formally as CH_3^+ in biological systems) as direct donors for the CH_3^- unit.[14] It is, however, assumed that the methyl group of L-methionine is transferred possibly with a corrin as an intermediate carrier (reverse formation[15] of L-methionine from methylcobalamin and homocysteine?) and from there as CH_3^- to phosphonoacetaldehyde (**3**). Seto et al. showed that the methyl group of [^{14}C-methyl]methylcobalamin is incorporated into fosfomycin by a growing culture of *Streptomyces wedmorensis*.[5] It is, however, in no way shown that this is the direct donor for the methyl group.

The two experiments described add further support to the assumption that in the biosynthesis of fosfomycin a methyl group originating from L-methionine adds to the carbonyl group of phosphonoacetaldehyde as CH_3^- by means of an umpolung step which has, until recently, been unknown. Experiments are currently underway to ascertain whether a chiral methyl group is transferred with retention or inversion of configuration.

Experimental Procedure

8: To a solution of **7** (5.08 g, 20 mmol) in $H_2{}^{18}O$ (95% ^{18}O; 1.0 g, 50 mol) was added chlorotrimethylsilane (0.51 mL, 4 mmol), and the solution was stirred for 72 h at room temperature. The mixture was then taken up in 40 mL anhydrous ethanol (pretreated with sodium) to which $NaBH_4$ (0.756 g, 20 mmol) was added in portions (exothermic reaction). After one hour glacial acetic acid (0.57 mL, 10 mmol) was added. The solution was concentrated and the residue dissolved in 30 mL water. Mannitol (4.0 g) was added and the solution was continuously extracted with ethyl acetate for 2 h. Purification by flash chromatography (eluting first with ethyl acetate, then ethyl acetate/ethanol 10/1; R_f **8** = 0.49 (in ethyl acetate/ethanol 10/1), visualized with I_2) and Kugelrohr distillation (90–100 °C/8 Torr) gave 2.0 g (54% yield) of **8** as a colorless oil. IR (film) [cm^{-1}]: $\nu = 3374$ (OH); ^{1}H NMR (400.1 MHz, $CDCl_3$, TMS): $\delta = 4.12$ (m, 4H; $2\times POCH_2$), 3.88 (dt, ^3J(P,H) = 18.7 Hz, ^3J(H,H) = 6.4 Hz, 2H; CH_2OH), 3.48 (br. s, 1H, OH), 2.07 (dd, ^2J(P,H) = 17.7 Hz, ^3J(H,H) = 6.4 Hz, 2H; PCH_2), 1.34 (t, ^3J(H,H) = 6.9 Hz, 6H; $2\times CH_3$); $^{13}C\{^1H\}$ NMR (100.6 MHz, $CDCl_3$): $\delta = 61.694$ (d, ^2J(P,C) = 6.5 Hz; $POCH_2$), 56.435 (d, ^2J(P,C) = 4.6 Hz, C-^{16}O; d for C-^{18}O shifted upfield by $\Delta\delta = 0.018$), 29.069 (d, ^1J(P,C) = 138.0 Hz; PCH_2), 16.259 (d, ^3J(P,C) = 3.0 Hz; CH_3).

9: A solution of **8** (1.83 g, 10 mmol), bromotrimethylsilane (5.2 mL, 40 mmol), and allyltrimethylsilane (3.2 mL, 20 mmol) in anhydrous CCl_4 (20 mL) was heated for 3 h at 50 °C with exclusion of moisture. Volatile components were removed in vacuo (0.2 Torr, up to 40 °C). The residue was dissolved in 15 mL ethanol and 15 mL water and concentrated after 10 min. Water was added again twice and the solution concentrated. Finally the residue was dissolved in 30 mL water and lyophilized; 1.2 g (94% yield). The viscous oil was used directly for the feeding experiments. ^{1}H NMR (400.1 MHz, D_2O): $\delta = 3.95$ (dt, ^3J(P,H) = 12.3 Hz, ^3J(H,H) = 7.4 Hz, 2H; CH_2OH), 2.20 (dt, ^3J(P,H) = 18.2 Hz, ^3J(P,H) = 7.4 Hz, 2H; PCH_2).

The feeding experiment with L-[methyl-$^2H_3$]methionine (300 mg L^{-1}, 99% D) and the isolation of labeled **6** were carried out according to literature procedures [9]. Six 1 L Erlenmeyer flasks, each with 220 mL medium were used. **6** was isolated by ion-exchange chromatography (Dowex 50, H^+; Dowex 50, H^+; Dowex 1, OAc; Dowex 50 H^+, 1 N HCO_2H). Yield after crystallization: 5.5 mg.

Feeding experiment with **9**: The free acid was added to the main culture before autoclaving (470 mg L^{-1}); 2 × 6 Erlenmeyer flasks. The eluates of the first Dowex-50 H^+ column were combined for further workup. Yield: 8 mg labeled **6**.

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