Biosynthesis of Natural Products with a P–C Bond. Part 6.[1] Preparation of Deuterium- and Carbon-13-Labelled L-Alanyl- and L-Alanyl-L-alanyl-(2-aminoethyl)phosphonic Acids and their Use in Biosynthetic Studies of Fosfomycin in *Streptomyces fradiae*

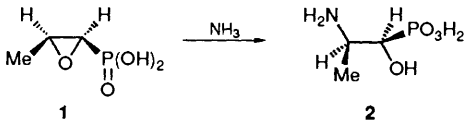
Friedrich Hammerschmidt,*[a] Hanspeter Kählig[a] and Norbert Müller[b]
[a] *Institut für Organische Chemie der Universität Wien, Währingerstrasse 38, A-1090 Wien, Austria*
[b] *Institut für Chemie, Johannes-Kepler-Universität, Altenbergerstrasse 69, A-4040 Linz, Austria*

(2-Aminoethyl)phosphonic acid (AEP) is not taken up by *Streptomyces fradiae* in the biosynthesis of fosfomycin. Attachment of L-alanine or L-alanyl-L-alanine to the amino group of deuteriated AEP affords di- and tri-peptides **4** and **5** which are transported into the cell.[2-^{13}C]AEP is synthesized from Na^{13}CN and the tosyloxymethylphosphonate **7**. It is transformed into dipeptide **4d**, which acts as a carrier for [2-^{13}C]AEP which is incorporated into fosfomycin with an enrichment of 2%.

Fosfomycin **1** is a structurally simple, clinically used antibiotic discovered in the culture broth of strains of *Streptomyces*.[2,3] It is a member of a small, steadily growing number of natural products containing a P–C bond.[4] The biosynthesis and stereochemical aspects thereof are still largely unknown. It is assumed that the P–C bond of fosfomycin is formed by intramolecular rearrangement[5,6] of a phosphoenol pyruvate to phosphonopyruvic acid. The enzyme effecting this transformation has been isolated from protozoa[7] containing (2-aminoethyl)phosphonic acid (AEP) and partially purified from *Streptomyces hygroscopicus* strains producing bialaphos.[8] The PC_3-unit is decarboxylated to give phosphonoacetaldehyde. Mutants of *Streptomyces wedmorensis* incorporate (2-aminoethyl)- and (2-hydroxyethyl)-phosphonic acid into fosfomycin, presumably *via* phosphonoacetaldehyde.[9] The methyl group[6] of fosfomycin derives from L-methionine acting as a donor of formal 'CH_3^+' after transformation into *S*-adenosyl-L-methionine. For polarity reasons a direct methylation of the positively polarized carbonyl carbon of phosphonoacetaldehyde is precluded. In our previous paper[1] it was shown that di-^{18}O-oxygen does not label the oxirane oxygen of fosfomycin, thus excluding (*Z*)-prop-1-enylphosphonic acid as an intermediate in the synthesis of fosfomycin.

Results and Discussion

[1,1-$^2H_2$]- and [2,2-$^2H_2$]-(2-Aminoethyl)phosphonic acid {abbreviated [1,1-$^2H_2$][10]- and [2,2-$^2H_2$]-AEP[11]} and all four chirally monodeuteriated AEPs have been described[12] and could be used as stereochemical probes. As the mutant strain of *Streptomyces wedmorensis* was not available, a mixture of [1,1-$^2H_2$]- and [2,2-$^2H_2$]-AEP (1:1) was added to the growth medium for a shake culture of *Streptomyces fradiae*, which also produces fosfomycin. Fosfomycin in the culture broth was transformed by ammonia into the aminophosphonic acid **2**, which was isolated by ion-exchange chromatography [eqn. (1)].[1]



1 2 (1)

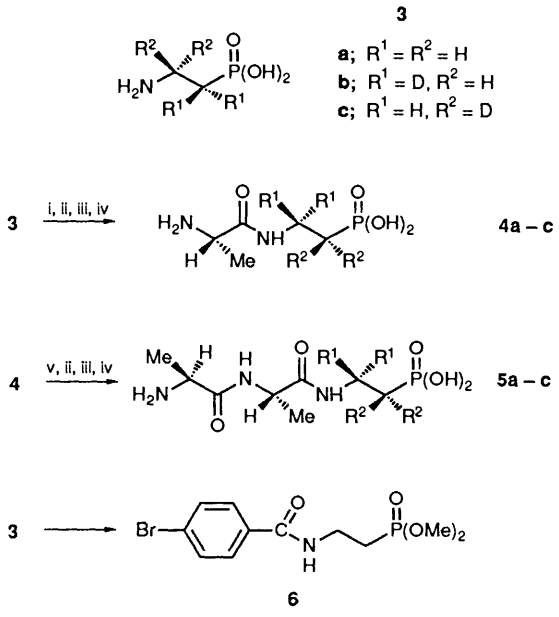
The amount of deuterium at C-1 and C-2 of compound **2**, if present at all, is below the detection limit as judged indirectly by ^{1}H NMR spectroscopy. The wet cell mass was washed three times with sterile 0.8% saline to remove [1,1-$^2H_2$]AEP from the medium and was hydrolysed in refluxing 6 mol dm^{-3} hydrochloric acid and worked up for AEP.[10] No AEP could be detected either by paper chromatography or by ^{1}H NMR spectroscopy of fractions from ion-exchange chromatography normally containing AEP. These findings were taken as evidence that AEP is not taken up by *Streptomyces fradiae* and therefore cannot possibly be incorporated into fosfomycin.

It turned out to be necessary to transform AEP into a compound which is taken up by the cell and degraded inside the cell to release AEP. Attachment of one or two amino acids to an impermeable molecule is used to induce illicit transport by di- and oligo-peptide permeases. This principle was used first to smuggle histidinol phosphate ester, a biosynthetic intermediate, into bacteria as its glycyl-glycyl derivative.[13] The uptake of L-ala(P),[14] the phosphonic acid analogue of alanine, and other compounds[15] is brought about by a dipeptide or higher peptide mimetic to enable transport into the cell to occur.

This principle was tried here by attaching L-alanine and L-alanyl-L-alanine to the amino group of AEP by a peptide bond. Reaction of an unprotected aminophosphonic acid with a *N*-protected, carboxy-activated amino acid is a low-yield process.[16] The use of esters[17] of aminophosphonic acids gives higher yields of peptide, but the protection and deprotection of the phosphonic acid group increases the number of steps.

Amino acids and peptides silylated under various conditions at the amino and carboxy groups can be made to react with activated esters such as mixed anhydrides, imidazolides, acid chlorides, and *p*-nitrophenyl esters of *N*-protected amino acids to form a peptide bond.[18] This reaction sequence may be carried out as a one-pot reaction and has already been used to prepare benzamides[11] of AEP and (2-amino-1-hydroxyethyl)phosphonic acid. *N*-Protected, activated esters of amino acids and silylated aminophosphonic acids reacted to give dipeptides in good yield.[19]

AEP **3a** was silylated in 5 min with chlorotrimethylsilane (3 mol equiv.) in dry, refluxing pyridine. Excess of the *N*-hydroxysuccinimide ester of benzyloxycarbony-L-alanine[20] was added at room temperature and the mixture was stirred for 18 h (Scheme 1). Removal of volatile material under reduced pressure, aqueous work-up, passage through Dowex 50WX4 (H^+-form), hydrogenolytic deprotection of the amino group, and ion-exchange chromatography of the crude product afforded dipeptide L-ala-AEP **4a** in 92% yield. L-Ala-AEP **4a** was treated in the same way as AEP, except that the reaction conditions for silylation were 60 °C for 10 min, and 24 h for



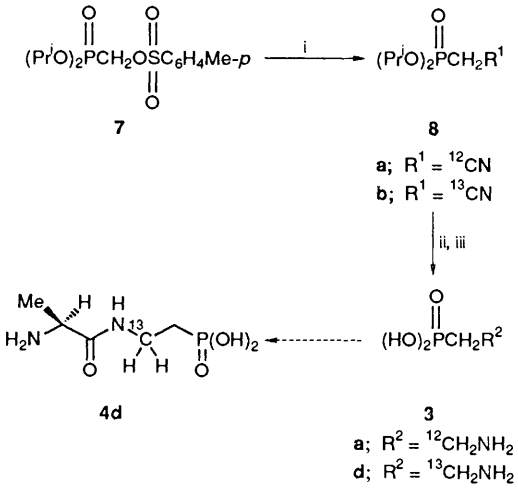
Scheme 1 *Reagents and conditions* (for **4a** and **5a**): i, **3a** (1.0 mol equiv.), Me_3SiCl (3.0 mol equiv.), refluxing pyridine, 5 min, room temp.; ii, Z-L-alanine *N*-hydroxysuccinimide ester (1.5 mol equiv.), 18 h; iii, Dowex 50WX8 (H^+); iv, 10% Pd/C/H_2, Dowex 1X8 (AcO^-) chromatography; v, **4a** (1 mol equiv.), Me_3SiCl (3.3 mol equiv.), pyridine, 60 °C, 10 min, room temp

amide formation. Tripeptide L-ala-L-ala-AEP **5a** was obtained in 85% yield. Racemization does not seem to occur as judged by ^1H NMR analysis of a sample of the mother liquor from crystallization of compound **5b**. No diastereoisomeric tripeptide could be detected. The di- and tri-peptides **4b**, **4c**, **5b** and **5c** containing [1,1-$^2H_2$]- and [2,2-$^2H_2$]-AEP were prepared similarly. Compounds purified by ion-exchange chromatography sometimes contained up to 2% of alanine, which was removed by recrystallization. This reaction sequence seems to be ideally suited to prepare di- and tri-peptides and possibly higher peptides containing aminophosphonic acids. The easily accessible *N*-hydroxysuccinimide ester of *N*-protected alanine turned out to be an excellent acylating agent.

Two feeding experiments were carried out. In the first experiment a mixture of L-ala-[1,1-$^2H_2$]AEP and L-ala-[2,2-$^2H_2$]AEP (1:1.6; concentration together 400 μg cm^{-3}) was added to the growth medium of *Streptomyces fradiae*. The amount of fosfomycin produced was the same (10 μg cm^{-3} culture broth) as without dipeptide. The cells harvested by centrifugation were washed three times with 0.8% sterile saline to remove any dipeptide present. The wet-cell mass was hydrolysed in refluxing hydrochloric acid and worked up for AEP as before. Crude AEP was derivatized[10] to the crystalline *p*-bromobenzamide **6** (Scheme 1). The isotopic composition as determined by mass spectroscopy was 0.5% d_0, 3.7% d_1 and 95.8% d_2. This result confirms the uptake of L-ala-AEP. The small, but significant, increase of d_1 from <2% for the starting material to 3.7% indicates that the peptide is at least partly cleaved enzymically to L-Ala and deuteriated AEP. Deuterium of [2,2-$^2H_2$]AEP[11] can be exchanged for hydrogen from the medium by a transaminase. The unlabelled material is endogenous AEP. The amount of deuteriated AEP present free or bound to L-ala in the cells as calculated from the chromatographically homogenous derivative **6** was 166 μg g^{-1} wet-cell mass. Fosfomycin was transformed into aminophosphonic acid **2**. Deuterium, if present at all, is below the detection limit [^1H NMR, no satellites (β-shift[21]) in the ^{13}C-{^1H}NMR spectrum for doublet (J_{PC} 153 Hz) at δ_C 73.00 for C-1 or doublet (J_{PC} 4 Hz) at δ_C 53.43 for C-2 for compounds labelled at C-2 or C-1, respectively]. One deuterium of [1,1-$^2H_2$]AEP at C-1 should be retained.[1] For mechanistic reasons it is not certain that the deuterium at C-2 of substrate **3c** on transformation into fosfomycin is incorporated into compound **2**.

The second feeding, with L-ala-L-ala-[1,1-$^2H_2$]AEP (400 μg cm^{-3}), was carried out under identical conditions. The isotopic composition of AEP isolated and derivatized was 1.3% d_0, 1.7% d_1 and 97% d_2. The amount of unlabelled material is larger, and the amount of singly labelled AEP is smaller, than before because an enzymic exchange of deuterium at C-1 for protium is less likely than at C-2. The concentration of free AEP in the cell could be as high as 27 μg g^{-1} cell mass. These two experiments demonstrate that di- and tri-peptides containing AEP are transported into *Streptomyces fradiae* and are cleaved by a peptidase. It is not clear whether the concentration of free AEP present in the cells is too low or whether a transaminase does not transform it efficiently to phosphonoacetaldehyde, the postulated intermediate in the biosynthesis of fosfomycin, to account for the incorporation, if at all, below the detection limit.

The use of deuterium suffers from low sensitivity. The incorporation of a few percent of deuterium into fosfomycin from a precursor cannot be detected. Therefore carbon-13 was used as a tracer. At first unlabelled sodium cyanide was treated in different solvents with the tosyloxymethylphosphonate **7** in the presence of a catalytic amount of sodium iodide (Scheme 2).



Scheme 2 *Reagents and conditions*: i, NaCN or $Na^{13}CN$ (99% ^{13}C), NaI (0.2 mol equiv. for 1 mol equiv. NaCN), DMF, 110 °C, 24 h; ii, BH_3·THF, 0 °C, 18 h, room temp.; iii, 6 mol dm^{-3} HCl, reflux, 18 h, ion-exchange chromatography

The isopropyl ester was chosen since this protecting group is more stable towards nucleophilic displacement than methyl or ethyl. In refluxing acetone only the corresponding iodide was formed; in propan-2-ol and dimethylformamide (DMF) at 80 °C the iodide was already partly displaced by cyanide. The best result was obtained in DMF at 110 °C and a reaction time of 24 h. The crude nitrile was reduced in tetrahydrofuran (THF) with diborane, deprotected by refluxing 6 mol dm^{-3} hydrochloric acid, and purified by ion-exchange chromatography to give AEP.[10] Repetition of these steps using $Na^{13}CN$ (99% ^{13}C) afforded [2-^{13}C]AEP **3d** in 54% yield with a chemical purity of 75%, which was transformed into L-ala-[2-^{13}C]AEP **4d** in 68% yield, and the product was fed to *Streptomyces fradiae* at a concentration of 300 μg cm^{-3} of medium. The ^{13}C-{^1H} NMR spectrum (100.6 MHz) of the corresponding product **2** showed a strongly enhanced signal for C-2 (δ_C 54.47, d, J_{PC} 4 Hz) with an enrichment for carbon-13 of 2%. This result is taken as evidence that the ^{13}C-labelled dipeptide is taken up into the cells, and that it is cleaved to L-alanine and [2-^{13}C]AEP which is transaminated to phosphonoacetaldehyde, a likely intermediate

Table 1 Analytical data for di- and tri-peptides **4** and **5**

Compound (Formula)	Yield (%)	M.p./°C (decomp.)	$[\alpha]_D^{20}$ (*c* 1.2 in 1 mol dm^{-3} HCl)	Found (%) (Required) C	H	D	N
4a	92	270–272	+2.4	30.4	6.5		14.1
($C_5H_{13}N_2O_4P$)				(30.62	6.68		14.28)
4b	98	271–276	+2.7	30.2	5.55	2.0	14.1
($C_5H_{11}D_2N_2O_4P$)				(30.31	5.60	2.03	14.14)
4c	86	274–275	+2.3	30.4	5.6	2.05	14.1
($C_5H_{11}D_2N_2O_4P$)				(30.31	5.60	2.03	14.14)
4d	68	268–270	+2.7				
5a	85	270–271	−43.1	36.0	6.8		15.4
($C_8H_{18}N_3O_5P$)				(35.96	6.79		15.73)
5b	88	275–277	−42.1	35.7	6.1	1.5	15.6
($C_8H_{16}D_2N_3O_5P$)				(35.69	5.99	1.50	15.61)
5c	98	275–277	−42.9	35.6	6.1	1.5	15.3
($C_8H_{16}D_2N_3O_5P$)				(35.69	5.99	1.50	15.61)

for fosfomycin. The low incorporation of AEP precludes the use of chirally deuteriated AEP to study stereochemical aspects of the biosynthesis. It will be shown in a forthcoming paper that (2-hydroxyethyl)phosphonic acid is incorporated much more efficiently into fosfomycin than is AEP.

Experimental

For general points see ref. 1; a modified Bruker WM 360 with ASPECT 3000 Computer and digital phase-shifters (90 MHz ^{13}C frequency) was used to obtain some of the ^1H and ^{13}C NMR spectra. *J*-values are given in Hz.

Preparation of N-L-*Alanyl-(2-aminoethyl)phosphonic Acid* (L-*ala-AEP*) **4a**—AEP[22] (0.5 g, 4 mmol) and chlorotrimethylsilane (1.304 g, 12 mmol) were stirred and refluxed under argon in dry pyridine (20 cm^3) for 5 min, and then allowed to cool to room temperature. The *N*-hydroxysuccinimide ester of benzyloxycarbonyl-L-alanine[20] (1.92 g, 6 mmol) was added. After the mixture had been stirred for 18 h, volatile material was removed under reduced pressure (0.005 mmHg; 20 °C). Water and diethyl ether were added to the residue. The mixture was stirred for 10 min and filtered by suction. The aq. phase was extracted twice with diethyl ether, concentrated on a rotary evaporator, the residue was dissolved in water, and the solution was passed through Dowex 50 WX8 (H^+-form) (50–100 mesh; 50 cm^3) and eluted with water. The eluate was concentrated and the residue was hydrogenolysed on 10% palladium on carbon (300 mg) in a mixture of water and dry ethanol (40 cm^3: 60 cm^3) in a Parr hydrogenation apparatus at 3.4 atm for 2 h. The filtered solution was concentrated and purified by ion-exchange chromatography on Dowex 1X8 (AcO^--form) (100–200 mesh; column 1.2 × 65 cm).[11] Eight fractions (10 cm^3 each) were eluted with 0.001 mol dm^{-3} AcOH and ten with 0.1 mol dm^{-3} AcOH. Ninhydrin-positive fractions [paper chromatography (PC) with solvent and ninhydrin reagent as used for (2-amino-1-hydroxyethyl)phosphonic acid; R_F 0.44][11] were pooled and concentrated to give compound **4a**. An analytical sample was dissolved in hot water, precipitated by addition of ethanol, and dried *in vacuo* (0.001 mmHg; 50 °C; 2 h). Compounds **4b–4d** were prepared analogously. Yields and analytical data are given in Table 1.

Compound **4a**: ν_{max}(Nujol)/cm^{-1} 3320, 3300–2000br, 1667, 1538, 1205 and 1011; δ_H[250 MHz; D_2O; δ(HDO) 4.80] 1.49 (3 H, d, *J* 7.3, Me), 1.83 (2 H, dt, *J* 17.1 and 8, PCH_2), 3.42 (2 H, dt, *J* 11 and 8, $PCCH_2$) and 4.00 (1 H, q, *J* 7.3, NCH).

Compound **4b**: ν_{max}(Nujol)/cm^{-1} 3320, 3300–2000br, 1668, 1537, 1205, 1130, 1010 and 925; δ_H[250 MHz; D_2O; δ(HDO) 4.80] 1.48 (3 H, d, *J* 7, Me), 3.40 (2 H, d, *J* 11, $PCCH_2$) and 4.00 (1 H, q, *J* 7, CHN).

Compound **4c**: ν_{max}(Nujol)/cm^{-1} 3320, 3300–2000br, 1668, 1537, 1257, 1195, 1015 and 930; δ_H[250 MHz; D_2O; δ(HDO) 4.80] 1.47 (3 H, d, *J* 7, Me), 1.82 (2 H, d, *J* 17.2, PCH_2) and 4.02 (1 H, q, *J* 7, CHN).

Compound **4d**: ν_{max}(Nujol)/cm^{-1} 3323, 3300–2000br, 1667, 1537, 1202, 1125, 1024, 1012 and 930; δ_H[250 MHz; D_2O; DSS] 1.50 (3 H, d, *J* 6.9, Me), 1.82 (2 H, m, PCH_2), 3.41 (2 H, dq, *J* 142 and 9, $^{13}CH_2$) and 4.02 (1 H, q, *J* 6.9, CHN).

Preparation of Tripeptides.—*N*-L-*Alanyl*-L-*alanyl*-(*2-aminoethyl*)*phosphonic acid* **5a**.—Dipeptide **4a** (0.425 g, 2.14 mmol) and chlorotrimethylsilane (0.77 g, 7.1 mmol) were stirred and heated at 60 °C for 10 min in dry pyridine (15 cm^3) under argon. After the mixture had cooled to room temperature the *N*-hydroxysuccinimide ester of benzyloxycarbonyl-L-alanine (1.03 g, 3.2 mmol) was added and the mixture was stirred for 24 h. Work-up and purification followed the procedure for compound **4a**, except that the residue which was passed through Dowex 50 (H^+) had to be dissolved in water (50 cm^3), and the material for hydrogenolysis had to be dissolved in a mixture of water and dry ethanol (30 cm^3:70 cm^3). PC of product **5a**: R_f 0.44. Compounds **5b** and **5c** were prepared similarly.

Compound **5a**: ν_{max}(Nujol)/cm^{-1} 3285, 3263, 3200–2000br, 1644, 1518, 1170, 1125, 1022 and 927; δ_H[250 MHz; D_2O; δ(HDO) 4.80] 1.36 and 1.54 (each 3 H, 2 d, *J* 6.9, 2 × Me; irradiation at δ 1.36 causes collapse of q at δ 4.26 to s), 1.82 (2 H, dt, *J* 17.9 and 8.4, PCH_2), 3.40 (2 H, q, *J* 8.4, CH_2N) and 4.06 and 4.26 (each 1 H, 2 q, *J* 6.9, 2 × CHN).

Compound **5b**: ν_{max}(Nujol)/cm^{-1} 3285, 3267, 3200–2000br, 1643, 1517, 1169, 1128, 1064, 1017, 1002 and 947; δ_H[250 MHz; D_2O; δ(HDO) 4.80] 1.35 and 1.52 (each 3 H, 2 d, *J* 7, 2 × Me), 3.36 (2 H, *J* 10.8, CH_2N) and 4.05 and 4.24 (each 1 H, 2 q, *J* 7, 2 × CHN).

Compound **5c**: ν_{max}(Nujol)/cm^{-1} 3282, 3260, 3200–2000br, 1643, 1558, 1169, 1025, 1015 and 946; δ_H[250 MHz; D_2O; δ(HDO) 4.80] 1.37 and 1.53 (each 3 H, 2 d, *J* 7, 2 × Me), 1.80 (2 H, d, *J* 17.5, PCH_2) and 4.07 and 4.26 (each 3 H, 2 q, *J* 7, 2 × CHN).

Preparation of Compounds **3a** *and* **3d**.—Addition of 1,8-diazabicyclo[5.4.0]undec-7-ene (8 drops) to a stirred mixture of diisopropyl hydrogen phosphite[23] (10.0 g, 60.2 mmol) and paraformaldehyde (1.806 g, 60.2 mmol) at room temperature induced an exothermic reaction. When the reaction mixture had again reached room temperature (40 min), CH_2Cl_2 (70 cm^3) and 2 mol dm^{-3} HCl (4 cm^3) were added. The organic phase was separated and dried (Na_2SO_4). The solvent was removed and the oily residue was distilled (bulb-to-bulb) to give hydroxymethylphosphonic acid diisopropyl ester (10.8 g, 92%; compare preparation of hydroxymethylphosphonic acid diethyl ester[24]),

b.p. 105–113 °C at 0.005 mmHg; $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 3500 and 3300 (OH); δ_H(250 MHz; $CDCl_3$) 3.70 (1 H, br s, OH) and 3.86 (2 H, d, *J* 6.3, PCH_2). Tosyl chloride (6.673 g, 35 mmol) was added to a stirred, cooled (10 °C) solution of the above hydroxymethylphosphonate (6.87 g, 35 mmol) and triethylamine (7.1 g, 70 mmol) in dry CH_2Cl_2 (70 cm^3) under exclusion of moisture. After 1 h at 10 °C the mixture was stirred for 3 h at room temperature, then extracted successively with 2 mol dm^{-3} HCl (2 × 30 cm^3), water, and saturated aq. $NaHCO_3$, and was dried (Na_2SO_4). The solvent was removed and the residue was purified by flash chromatography on silica gel (250 g) with CH_2Cl_2–ethyl acetate (10:1) (R_f 0.5) as eluent to give the tosyloxyphosphonate **7** (11.5 g, 94%), $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 2985, 1375, 1192, 1180 and 1005; δ_H(250 MHz; $CDCl_3$) 4.12 (2 H, *J* 10.3, PCH_2).

Compound **7** (2.1 g, 6 mmol), NaI (0.15 g, 1 mmol), and NaCN (0.245 g, 5 mmol) were stirred in dry DMF (15 ml) at 110 °C (bath temperature) under argon for 24 h. The solvent was removed under reduced pressure (0.1 mmHg; 40 °C). Water was added to the residue and cyanomethylphosphonate **8a** was continuously extracted with diethyl ether for 2 h. The extract was evaporated. Toluene was added to the oily residue and then evaporated off. The residue was dried at 0.005 mmHg at 40 °C and gave crude phosphonate **8a** (0.88 g, 86%, purity *ca.* 70%); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 2257 (CN); δ_H (250 MHz; $CDCl_3$) 2.85 (2 H, d, *J* 20.5, CH_2CN); DMF, educt, and iodomethylphosphonate [3.01 (d, *J* 10.5, CH_2I)] were also present.

Crude phosphonate **8a** (0.74 g, 2.5 mmol, purity 70%) was dissolved in dry THF (10 cm^3) under argon. BH_3·THF (15 cm^3; 1 mol dm^{-3}) was added dropwise to the stirred, cooled solution at 0 °C. After being stirred for 18 h at room temperature the solution was cooled to −50 °C and a solution of MeOH (2 cm^3) in THF (5 cm^3) was added slowly, followed by HCO_2H (2 cm^3) 15 min later. The mixture was stirred for another 30 min, then at room temperature until evolution of hydrogen ceased (*ca.* 30 min). The solvent was evaporated off, and the residue was refluxed in 6 mol dm^{-3} HCl (60 cm^3) for 18 h and then purified by ion-exchange chromatography to give AEP (0.28 g, 90%), identical (PC) with an authentical sample.

Preparation of [2-^{13}C]*AEP* **3d**.—Compound **7** (4.2 g, 12 mmol), NaI (0.3 g, 2 mmol), and $Na^{13}CN$ (0.5 g, 10 mmol; 99% ^{13}C) were treated as for compound **8a** to yield crude cyano phosphonate **8b** (2.63 g; 60% purity); $\nu_{max}(CH_2Cl_2)/cm^{-1}$ 2205 (^{13}CN); δ_H(250 MHz; $CDCl_3$) 2.83 (2 H, dd, *J* 20.8 and 10.6, $CH_2{}^{13}CN$).

The crude product **8b** was reduced with BH_3·THF (30 cm^3), deprotected, and purified as for compound **3a** to give [2-^{13}C]AEP **3d** (0.684 g, 54%; chemical purity 75%). An analytical sample was recrystallized from aq. ethanol, m.p. 271–273 °C (decomp.) (lit.,[22] 250 °C to 296–299 °C for AEP); ν_{max} (Nujol)/cm^{-1} 3700–2000br, 1655, 1556, 1281, 1165, 1143, 1035, 1007 and 910; δ_H(250 MHz; D_2O; DSS) 1.98 [2 H, ddt, *J* 18.2 and 8.0, *J*(^{13}C, H) 5.9, 1-H] and 3.22 [ddt, *J* 10.5 and 8.0, *J*(^{13}C, H) 145, 2-H]. Crude product **3d** (0.43 g) was transformed into dipeptide **4d**.

Feeding Experiments, Isolation of Compound **2** *from Culture Broth,*[1] *and Isolation*[10,11] *of Compound* **3** *from Wet-cell Mass.*—*Streptomyces fradiae* was shake cultured in three 1000 cm^3 Erlenmeyer flasks containing corn-starch medium (220 cm^3).[1] Compounds to be tested for incorporation were added to the medium before sterilization. The concentration of fosfomycin was determined microbiologically to be *ca.* 10 μg cm^{-3} culture broth after centrifugation of cells. Pooled cells were washed three times with sterile saline (0.8%; total of 500 cm^3), hydrolysed for 24 h with refluxing water (200 cm^3)–10 mol dm^{-3} HCl (300 cm^3), and worked up for AEP. The Dowex 50 (H^+) column was eluted with 0.5 mol dm^{-3} HCl (400 cm^3) and the Dowex 1 (AcO^-) column with 0.002 mol dm^{-3} AcOH. Fractions containing AEP as determined by PC were pooled, concentrated, and derivatized.[10] Fosfomycin in the culture broth was transformed into amino phosphonic acid **2** and isolated by ion-exchange chromatography.[1] The mixture of compounds **2** and **3** (or **4**, or **5**) obtained after ion-exchange chromatography on Dowex 1 (AcO^-) were separated on a Dowex 50 (H^+) column with 1 mol dm^{-3} HCO_2H as eluent.

Feeding of [1,1-$^2H_2$]- *and* [2,2-$^2H_2$]-*AEP* (1:1; *total* 0.2 mg cm^{-3} *of medium*). Wet-cell mass (30.0 g) containing no AEP; compound **2** (2.5 mg).

Feeding of a mixture of L-*ala-AEP* **4b** *and* **4c** (1:1.6; 0.4 mg cm^{-3}). Wet-cell mass (41.0 g), one half of material containing AEP was treated[10] to give chromatographically homogenous compounds **6** (9 mg) and **2** (2.5 mg).

Feeding of L-*ala*-L-*ala*-[1,1-$^2H_2$]*AEP* **5b** (0.4 mg cm^{-3}). Wet-cell mass (41.0 g), one half of material containing AEP was transformed into compounds **6** (2.5 mg) and **2** (2 mg).

Feeding of L-*ala*-[2-^{13}C]*AEP* (0.3 mg cm^{-3}). Six Erlenmeyer flasks were used; compound **2** (5 mg) was isolated.

Acknowledgements

F. H. thanks Prof. Dr. E. Zbiral for support, the Fonds zur Förderung der wissenschaftlichen Forschung (Vienna) for a grant (P 7183-CHE), and for financing (in part) the NMR spectrometers (project numbers 4009 and 6537C).

References

1 Part 5, F. Hammerschmidt, G. Bovermann and K. Bayer, *Liebigs Ann. Chem.*, 1990, 1055.
2 D. Hendlin, E. O. Stapley, M. Jackson, H. Wallick, A. K. Miller, F. J. Wolf, T. W. Miller, L. Chaiet, F. M. Kahan, E. L. Foltz, H. B. Woodruff, J. M. Mata, S. Hernandez and S. Mochales, *Science*, 1969, **166**, 122; F. M. Kahan, J. S. Kahan, P. J. Cassidy and H. Kropp, *Ann. N.Y. Acad. Sci.*, 1974, **235**, 364.
3 B. G. Christensen, W. J. Leanza, T. R. Beattie, A. A. Patchett, B. H. Arison, R. E. Ormond, F. A. Kuehl, Jr., G. Albers-Schonberg and O. Jardetzky, *Science*, 1969, **166**, 123.
4 R. L. Hildebrand, *The Role of Phosphonates in Living Systems*, CRC Press, Boca Raton, Florida, 1983; P. Kafarski and P. Mastalerz, *Aminophosphonates—Natural Occurrence, Biochemistry and Biological Properties*, Beiträge zur Wirkstofforschung, Heft 21, eds. P. Oehme, H. Löwe and E. Göres, Akademie-Industrie-Komplex Arzneimittelforschung, DDR, Berlin, 1984; T. Hori, M. Horiguchi and A. Hayashi, *Biochemistry of Natural C–P Compounds*, Maruzen, Kyoto, 1984; L. Maier, *Phosphorus Sulfur*, 1983, **14**, 295; T. Hidaka, M. Mori, S. Imai, O. Hara, K. Nagaoka and H. Seto, *J. Antibiot.*, 1989, **42**, 491 and references cited therein.
5 W. A. Warren, *Biochim. Biophys. Acta*, 1968, **156**, 340.
6 T. O. Rogers and J. Birnbaum, *Antimicrob. Agents Chemother.*, 1974, **5**, 121.
7 E. Bowman, M. McQueney, R. J. Barry and D. Dunaway-Mariano, *J. Am. Chem. Soc.*, 1988, **110**, 5575; H, M. Seidel, S. Freeman, H. Seto and J. R. Knowles, *Nature*, 1988, **335**, 457; T. Takada and M. Horiguchi, *Biochim. Biophys. Acta*, 1988, **964**, 113; S. Freeman, H. M. Seidel, C. H. Schwalbe and J. R. Knowles, *J. Am. Chem. Soc.*, 1989, **111**, 9233.
8 T. Hidaka and H. Seto, *J. Am. Chem. Soc.*, 1989, **111**, 8012; T. Hidaka, M. Mori, S. Imai, O. Hara, K. Nagaoka and H. Seto, *J. Antibiot.*, 1989, **42**, 491.
9 S. Imai, H. Seto, H. Ogawa, A. Satoh and N. Otake, *Agric. Biol. Chem.*, 1985, **49**, 873; the wild-type organism will not give satisfactory results (personal communication from H. Seto).
10 F. Hammerschmidt, *Liebigs Ann. Chem.*, 1988, 531.
11 F. Hammerschmidt, *Liebigs Ann. Chem.*, 1988, 537.
12 F. Hammerschmidt, *Liebigs Ann. Chem.*, 1988, 955, 961.
13 B. N. Ames, G. F.-L. Ames, J. D. Joung, D. Tsuchiya and J. Lecocq, *Proc. Natl. Acad. Sci. USA*, 1973, **70**, 456.
14 J. G. Allen, F. R. Atherton, M. J. Hall, C. H. Hassall, S. W. Holmes, R. W. Lambert, L. J. Nisbet and P. S. Ringrose, *Nature*, 1978, **272**, 56.

15 H. Diddens, H. Zähner, E. Kraas, W. Göhring and G. Jung, *Eur. J. Biochem.*, 1976, **66**, 11; S. M. Hammond, A. Claesson, A. M. Jansson, L.-G. Larsson, B. G. Pring, C. M. Town and B. Ekström, *Nature*, 1987, **327**, 730.
16 P. Kafarski, B. Lejczak and P. Mastalerz, *Phosphonopeptides—Synthesis and Biological Activity*, Heft, 25, Beiträge zur Wirkstoffforschung, eds. P. Oehme, H. Löwe and E. Göres, Akademie-Industrie-Komplex, Arzneimittelforschung, DDR, Berlin, 1985.
17 P. Kafarski and B. Lejczak, *Synthesis*, 1988, 307.
18 L. Birkofer, A. Ritter and P. Neuhausen, *Liebigs Ann. Chem.*, 1962, **659**, 190; L. Birkofer and A. Ritter, *Angew. Chem., Int. Ed. Engl.*, 1965, **4**, 417; H. R. Kricheldorf, *Liebigs Ann. Chem.*, 1972, **763**, 17.
19 P. Kafarski, M. Soroka and B. Lejczak, in *Peptide Chemistry*, eds. T. Shiba and S. Sakakibara, Protein Research Foundations, Osaka, 1988, p. 307.
20 G. W. Anderson, J. E. Zimmerman and F. M. Callahan, *J. Am. Chem. Soc.*, 1964, **86**, 1839.
21 M. J. Garson and J. Staunton, *Chem. Soc. Rev.*, 1979, **8**, 539; J. C. Vederas, *Nat. Prod. Rep.*, 1987, **4**, 277.
22 A. F. Isbell, J. P. Berry and L. W. Tansey, *J. Org. Chem.*, 1972, **37**, 4399.
23 K. Sasse in *Methoden der Organischen Chemie (Houben-Weyl), Organische Phosphorverbindungen*, Vol. XII/2, ed. Eugen Müller, Georg Thieme Verlag, Stuttgart, 1964, p. 23.
24 A. F. Kluge in *Org. Synth.*, 1986, **64**, 80.

Paper 0/02969F
Received 2*nd July* 1990
Accepted 28*th September* 1990