Indirect Evidence for the Biosynthesis of (1*S*,2*S*)-1,2-Epoxypropylphosphonic Acid as a Co-Metabolite of Fosfomycin [(1*R*,2*S*)-1,2-Epoxypropylphosphonic Acid] *by Streptomyces fradiae*[‡]

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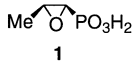
Keywords: Fosfomycin / Antibiotics / Electrophoresis / Natural products

Treatment of the culture broth of fosfomycin (**1**) producing *Streptomyces fradae* with ammonia gives 2–3% of the C-1 epimeric compound **5**, as well as the known (1*R*,2*R*)-2-amino-1-hydroxypropylphosphonic acid (**3**) derived from fosfomycin. The configuration of **5** was determined by capillary electrophoresis employing a quinine carbamate-type chiral selector and by synthesis from a monoprotected 1,2-dihydroxypropylphosphonate of known absolute configuration. It is postulated that (1*S*,2*R*)-2-amino-1-hydroxypropylphosphonic acid (**5**) is derived by ring opening of a *trans*-epoxide, formed as a co-metabolite of fosfomycin (*cis*-epoxide), with ammonia.

Introduction

Fosfomycin (**1**), which is produced by various *Streptomyces* species,[2] *Pseudomonas syringae*[3] and *Pseudomonas viridiflava*,[4] is a clinically used antibiotic that blocks the first step of the bacterial cell wall biosynthesis.[5]



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As shown by feeding experiments with labelled intermediates,[1,6–8] as well as by use of mutants,[9] the biosynthesis of the oxirane ring is unique. Compound **1** is produced by oxidative cyclisation of (*S*)-2-hydroxypropylphosphonic acid (**2**), the oxirane oxygen atom being derived from the oxygen atom of the hydroxyl group at C-2.[8,9] Furthermore, the C–H bond at C-1 is replaced by the O–C bond, with net inversion of the configuration, as proven by use of deuterated precursors that are chiral at C-1.[7] Very recently, Liu and co-workers have purified the epoxidase that catalyses the formation of fosfomycin (**1**).[10] This paper prompted us to report our independent findings.

Results and Discussion

In our biosynthetic studies, we did not isolate fosfomycin (**1**), which is a highly polar and labile compound, but the (1*R*,2*R*)-2-amino-1-hydroxypropylphosphonic acid (**3**), formed by ring opening of fosfomycin (**1**), along with the isomeric (1*S*,2*S*)-1-amino-2-hydroxypropylphosphonic acid (**4**) (Scheme 1).[6] The crude product **3** (about 5 mg from 1.32 L of broth) obtained by ion exchange chromatography was further purified by crystallisation. The ^{1}H and ^{31}P NMR spectra[11] of a crude sample of **3** in D_2O showed additional signals of a small amount (2–3%) of an unknown compound **5** along with the signals for **3**. On the basis of the ^{1}H and ^{31}P NMR spectra and the similar behaviour of **5** and **3** during ion exchange and paper chromatography, we assumed that **5** was an isomer of **3** epimeric at C-1 or C-2. This assumption was proven by the ^{1}H and ^{31}P NMR spectra of pure (±)-(1*R**,2*S**)-2-amino-1-hydroxypropylphosphonic acid[12] alone and in admixture with authentic **3**. The aminophosphonic acid **5** could also be detected in larger quantities in different mother liquors kept for years after crystallisation of **3**. Compound **5** is either a by-product of the treatment of fosfomycin with ammonia or the reaction product of the opening of a *trans*-epoxide formed in small quantities during biosynthesis as a co-metabolite along with fosfomycin (*cis*-epoxide). In the latter case, the occurrence of compound **5** could give a clue to the formation of the oxirane ring of fosfomycin (**1**).

To check whether the unknown compound results from the reaction of fosfomycin (**1**) with ammonia, we heated 1.0 g of fosfomycin (**1**) with concentrated aqueous ammonia

[‡] Biosynthesis of Natural Products with a P–C Bond, 12. – Part 11: Ref.[1]

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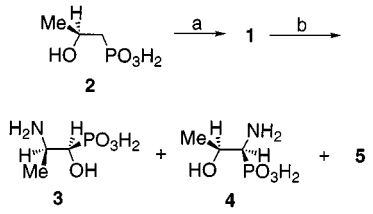


Scheme 1. Reagents: (a) *Streptomyces fradiae*; (b) H_2O/NH_3/60 °C

at 60 °C for three days.[6] Both amino-hydroxypropylphosphonic acids **3** and **4** were isolated by an improved procedure. They were adsorbed on Dowex 50, H^+, eluted with water and investigated by NMR spectroscopy. The (1*R*,2*R*)-2-amino-1-hydroxypropylphosphonic acid (**3**) being eluted second did not contain compound **5**. Consequently, **5** is not a side product of the ring opening of fosfomycin (**1**) with ammonia.

The determination of the absolute configuration of **5** in admixture with **3** was a major challenge. Previously, Lindner et al. have demonstrated that all eight stereoisomeric 1,2-amino-hydroxypropylphosphonic acids can be separated as their *N*-(2,4-dinitrophenyl) derivatives in one run by stereoselective capillary electrophoresis using a quinine carbamate-type chiral selector (see **A** in Figure 1).[13] The elution order was deduced in part by using amino-hydroxypropylphosphonic acids of known absolute configuration and in part by comparison with the corresponding carboxylic acids. To assign the peaks exclusively on the basis of authentic 1,2-amino-hydroxypropylphosphonic acids, two more, the (1*R*,2*S*)-1-amino-2-hydroxypropylphosphonic acid (**8**) and the (1*S*,2*R*)-2-amino-1-hydroxypropylphosphonic acid (**11**) were prepared (Scheme 2). Diisopropyl (1*S*,2*S*)-2-benzyloxy-1-hydroxypropylphosphonate (**6**) obtained by the base-catalysed addition of diisopropyl phosphite to (*S*)-*O*-benzyl lactaldehyde[14] was used as the common starting material. A Mitsunobu reaction with $Ph_3P/DEAD/HN_3$ gave azide **7**, which was reduced, deblocked and purified by ion exchange chromatography to give the aminophosphonic acid (1*R*,2*S*)-**8**.[15]

To prepare the 2-aminophosphonic acid **11** from **6**, the hydroxyl group was protected with *t*BuMe$_2$SiCl/imidazole in dry DMF at 40 °C for three days to give the silyl ether **9** in 71% yield. A small amount of a by-product with (*R*)-configuration at C-1 was also formed, which increased to 10% when the reaction temperature was 60 °C.[16,17] This compound was not fully characterised and may be generated by an imidazole-catalysed elimination of phosphite from **6** and readdition to the aldehyde thus formed to give either **6** or its epimer, followed by silylation. Alternatively, **9** could decompose thermally to a silylated phosphite and aldehyde, which on readdition produce **9** or its epimer. Hydrogenolytic removal of the benzyl group from **6** generated the alcohol, which was directly transformed into the 2-azido phosphonate **10** in 58% overall yield by use of the Mitsunobu reaction as before. The azide was reduced, deprotected and purified to give the 2-aminophosphonic acid **11** in 68% yield. Both aminophosphonic acids **8** and **11** were

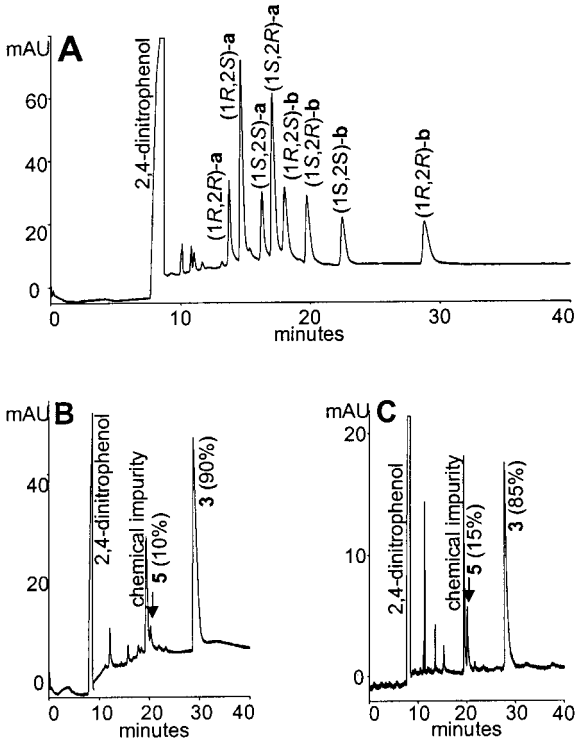
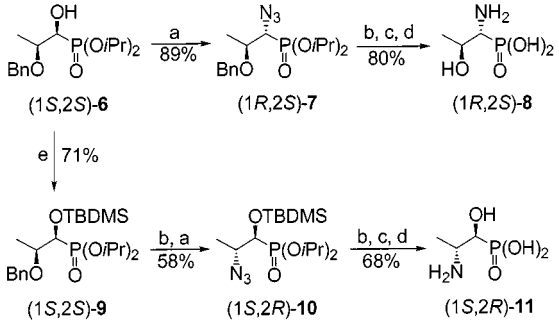


Figure 1. **A**: CE of *N*-(2,4-dinitrophenyl) derivatives of a mixture of four stereoisomeric 1-amino-2-hydroxypropylphosphonic acids (**a**) and four stereoisomeric 2-amino-1-hydroxypropylphosphonic acids (**b**); (1*R*,2*R*)-**b** = **3**, (1*S*,2*R*)-**b** = **5**. **B**: Derivatised sample of **3** and **5** of biological origin, part of **3** being removed by crystallisation to enrich the mother liquor in **5**. **C**: **B** spiked with (1*S*,2*R*)-**11** = **5**



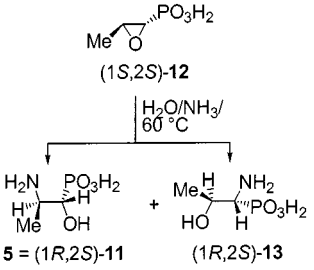
Scheme 2. Reagents: (a) $Ph_3P/DEAD/HN_3$; (b) $Pd/C/H_2$; (c) 6 N HCl/reflux; (d) Dowex 50, H^+; (e) *t*BuMe$_2$SiCl/imidazole/DMF/40 °C

pure diastereomers with enantiomeric excesses of ≥98% as determined by capillary electrophoresis (CE) with a quinine carbamate-type chiral selector. With these two additional samples in hand, the absolute configuration of all eight peaks could be assigned on the basis of 1,2-amino-hydroxypropylphosphonic acids of known absolute configuration. The result supports the previous assignments based on comparison with the corresponding amino carboxylic acids.[13]

The reaction mixture of fosfomycin (**1**) with aqueous ammonia was concentrated in vacuo. A sample of the res-

idue was derivatised with Sanger's reagent in a buffer system and investigated by CE. The ratio of the aminophosphonic acids **3** and **4** derived[6] from fosfomycin (**1**) was 73:26. The aminophosphonic acid **5** could not be detected. Finally, two samples of **3** of biological origin were crystallised to enrich **5** in the mother liquor and investigated similarly. The ratios of **3** and **5** were 90:10 (see **B** in Figure 1) and 86:14, respectively. The absolute configuration of the 2-amino-1-hydroxypropylphosphonic acid **5** is 1*S*,2*R* based on retention time; this was confirmed independently by CE of a sample spiked with (1*S*,2*R*)-**11** (see **C** in Figure 1).

This allows us to deduce the absolute configuration of the *trans*-1,2-epoxypropylphosphonic acid (**12**) which is the postulated precursor of **5** (Scheme 3). As ring opening of the epoxide with ammonia occurs by inversion of the configuration at C-2, **12** must have a 1*S*,2*S* configuration. It is likely that ammonia also attacks at C-1 to give the 1-aminophosphonic acid (1*R*,2*S*)-**13**, which is removed on isolation of the mixture of **3** and **5** by ion-exchange chromatography.



Scheme 3. Reagents: (a) H_2O/NH_3/60 °C

Isolation of the *trans*-epoxide (1*S*,2*S*)-**12**, the unambiguous proof of its occurrence in the culture broth, would, at best, be extremely difficult. As the *trans*-epoxide is as polar as fosfomycin (**1**), the same difficulties in isolation would result:[18] its low concentration in the culture broth — 2−3% of that of fosfomycin (**1**) — and the problems of separation from fosfomycin, aggravate the problems associated with the purification of the *trans*-epoxide. Not surprisingly, we did not try to isolate it.

Based on biosynthetic considerations and on the recent report on the enzyme catalysing the oxidative cyclisation of (*S*)-2-hydroxypropylphosphonic acid (**2**) to fosfomycin (**1**), the detection of 2-amino-1-hydroxypropylphosphonic acid **5** is not unexpected. Compound **5** is strong evidence for a radical intermediate generated from **2** by loss of hydrogen from C-1. The intermediate is transformed mainly into fosfomycin (**1**) and only traces of the *trans*-isomer occur after rotation about the C-1−C-2 bond. This has been proven by labelling experiments, and these results will be published separately.

Experimental Section

General: TLC: Merck precoated TLC plates (0.25 mm), silica gel 60, F_{254}. Spots on TLC plates were visualized by UV and/or dipping the plate into a solution of $[(NH_4)_6Mo_7O_{24}]\cdot 4H_2O$ (24.0 g) and $[Ce(SO_4)_2]\cdot 4H_2O$ (1.0 g) in 10% H_2SO_4 in water (500 mL), followed by heating with a hot gun. Spots of free aminophosphonic acids on TLC plates [silica, *i*PrOH/H_2O/conc. ammonia (6:3:1)] were visualized by dipping into a solution of 0.2% ninhydrin in 96% ethanol/acetic acid/2,4,6-trimethylpyridine (16:3:1) and heating with a hot gun. PC: Schleicher & Schüll 2043 Bmgl; solvent: *tert*-butyl alcohol/ethyl methyl ketone/formic acid/water 10:5:1:10. Aminophosphonic acids were visualized by spraying with or dipping into the solution of ninhydrin as used above and heating at 120 °C.[6] Flash chromatography: Merck silica gel 60, 0.040−0.063 mm. All starting materials were obtained from commercial suppliers and were generally used without further purification. IR: Perkin−Elmer FT 1600 IR-Spectrometer. The IR spectra of liquid samples were measured as films between NaCl plates or on a silicon disc (a solution of the sample in Uvasol $CHCl_3$ was applied to a Si plate and the solvent was allowed to evaporate).[19] The IR spectra of solids were measured as nujol mulls between NaCl plates. ^{1}H NMR and ^{13}C NMR (*J*-modulated): Bruker spectrometers AC 250F or AM 400 WB; TMS as internal standard. Optical rotation: Perkin−Elmer polarimeter 241 (1-dm cell). Melting points were measured with a Reichert Thermovar instrument and are uncorrected.

Diisopropyl (1*R*,2*S*)-1-Azido-2-benzyloxypropylphosphonate [(1*R*,2*S*)-7]: HN_3 (6 mL, 6.0 mmol, 1 M in toluene) was added to a stirred and cooled (0 °C) solution of hydroxyphosphonate (1*S*,2*S*)-**6** (1.32 g, 4 mmol) and PPh_3 (1.57 g, 6 mmol) in a mixture of dry toluene (35 mL) and dry CH_2Cl_2 (5 mL) under argon, followed immediately by DEAD (1.05 g, 0.95 mL, 6 mmol).[14] The flask was then heated at 40 °C until completion of the reaction (1 h, TLC: R_f = 0.62, CH_2Cl_2/EtOAc, 5:1). After addition of methanol (0.2 mL), the reaction mixture was concentrated in vacuo. The residue was purified by flash chromatography on silica (CH_2Cl_2/EtOAc, 5:1) to furnish the azide (1*R*,2*S*)-**7** (1.26 g, 89%) as a colourless oil. $[\alpha]_D^{20}$ = −28.56 (*c* = 2.50, CH_2Cl_2). IR (NaCl): $\tilde{\nu}$ = 3003, 2110, 1305, 990 cm^{-1}. ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.28, 1.32, 1.33, 1.34, 1.36 (five d, *J* = 7.0 Hz, each 3 H), 3.82 (dd, *J* = 3.5, 16.0 Hz, 1 H), 3.96 (m, 1 H), 4.57 (AB system, *J* = 12.0 Hz, 2 H), 4.76 (m, 2 H), 7.33 (m, 5 H). $C_{16}H_{26}N_3O_4P$ (355.38): calcd. C 54.08, H 7.37; found C 53.65, H 7.31.

(1*R*,2*S*)-1-Amino-2-hydroxypropylphosphonic Acid [(1*R*,2*S*)-8]: A solution of azide (1*R*,2*S*)-**7** (1.09 g, 3.07 mmol) in dry ethanol (70 mL) and concentrated HCl (1 mL) was hydrogenated over Pd/charcoal (300 mg, 10% Pd) in a Parr apparatus at a hydrogen pressure of 50 psi at room temperature for 18 h. The catalyst was removed by filtration. The filtrate was concentrated in vacuo and the residue dissolved in 6 N HCl (40 mL) and refluxed for 18 h. After cooling, the solution was concentrated in vacuo. The residue was dried in a vacuum dessicator over KOH and purified by ion exchange chromatography (Dowex 50, H^+, elution with water). The ninhydrin positive fractions (PC, R_f = 0.67) were pooled and concentrated in vacuo to furnish 1-aminophosphonic acid (1*R*,2*S*)-**8** (0.380 g, 80%); m.p. 221−223 °C (H_2O/ethanol, crystals were dried for 1 h at 75 °C/0.005 mm) [m.p. ref.[20] 217−219 °C]. $[\alpha]_D^{20}$ = −10.2 (*c* = 2.3, H_2O) {ref.[20] $[\alpha]_D^{20}$ = −8.9 (*c* = 0.294, H_2O)}. IR (Nujol): $\tilde{\nu}$ = 3000 (v. br.), 1630, 1520, 1200, 1080, 920 cm^{-1}. ^{1}H NMR (400.1 MHz, D_2O): δ = 1.27 (d, *J* = 6.5 Hz, 3 H), 3.35 (dd, *J* = 3.5, 15.0 Hz, 1 H), 4.25 (ddq, *J* = 3.5, 6.5, 10.5 Hz, 1 H). ^{13}C NMR (100.6 MHz, H_2O): δ = 17.46 (d, *J* = 1.5 Hz), 55.30 (d, *J* = 137.7 Hz), 65.09 (d, *J* = 2.3 Hz). ^{31}P NMR (162.0 MHz, H_2O): δ = 10.88. $C_3H_{10}NO_4P$ (155.09): calcd. C 23.23, H 6.50, N 9.03; found C 23.13, H 6.12, N 9.03.

Diisopropyl (1*S*,2*S*)-2-Benzyloxy-1-(*tert*-butyldimethylsiloxy)propylphosphonate [(1*S*,2*S*)-9]: A solution of 1-hydroxyphosphonate (1*S*,2*S*)-**6** (1.07 g, 3.25 mmol), imidazole (1.11 g, 16.25 mmol) and TBDMSCl (1.23 g, 8.13 mmol) in dry DMF (20 mL) was stirred under argon at 40 °C for three days. The solvent was then removed in vacuo (0.5 mm Hg, bath temperature not higher than 70 °C). The residue was then taken up in water and the product was extracted by washing twice with CH_2Cl_2. The combined organic layers were dried with Na_2SO_4 and concentrated in vacuo. The residue was purified by flash chromatography (CH_2Cl_2/EtOAc, 10:1, R_f = 0.39) to give the silyl ether (1*S*,2*S*)-**9** (1.02 g, 71%) as a viscous oil. $[\alpha]_D^{20}$ = +13.5 (*c* = 0.8, acetone). IR (Si): $\tilde{\nu}$ = 2977, 2930, 2858, 1253, 1107, 986 cm^{-1}. ^1H NMR (400.1 MHz, $CDCl_3$): δ = −0.08 (s, 3 H), 0.00 (s, 3 H), 0.77 (s, 9 H), 1.05 (d, *J* = 6.1 Hz, 3 H), 1.11 (d, *J* = 6.3 Hz, 3 H), 1.13 (d, *J* = 6.8 Hz, 3 H), 1.17 (d, *J* = 6.1 Hz, 6 H), 3.66 (m, 1 H), 3.74 (dd, *J* = 5.2, 8.5 Hz, 1 H), 4.44 (AB system, *J* = 11.7 Hz, 2 H), 4.52 (m, 1 H), 4.62 (m, 1 H), 7.17 (m, 5 H). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = −4.83, −4.66, 16.40 (d, *J* = 6.1 Hz), 18.33, 23.91 (d, *J* = 5.4 Hz), 24.05, 24.17 and 24.20 (three d, *J* = 5.4 Hz), 25.87, 70.43 (d, *J* = 7.7 Hz), 70.53 (d, *J* = 6.9 Hz), 71.43, 73.87 (d, *J* = 167.5 Hz), 75.78 (d, *J* = 5.4 Hz), 127.30, 127.90 (2C), 128.09 (2C), 138.80. ^{31}P NMR (162.0 MHz, $CDCl_3$): δ = 22.10. $C_{22}H_{41}O_5PSi$ (444.62): calcd. C 59.43, H 9.29; found C 59.30, H 9.05.

Diisopropyl (1*S*,2*R*)-2-Azido-1-(*tert*-butyldimethylsiloxy)propylphosphonate [(1*S*,2*R*)-10]: Silyl ether (1*S*,2*S*)-**9** (0.990 g, 2.226 mmol) was dissolved in dry ethanol (60 mL) and hydrogenated over Pd/charcoal (68 mg, 10% Pd) in a Parr apparatus at 50 psi at room temperature for 20 h. The catalyst was filtered off and the filtrate was concentrated in vacuo. The residue was dissolved twice in toluene and concentrated and dried to give the debenzylated phosphonate (0.711 g, 90%). It was transformed into the azide according to the procedure used for the preparation of (1*R*,2*S*)-**7**. After removing the solvent from the reaction mixture hexane was added to the residue and kept at 4 °C for 18 h. The crystalline precipitate was removed and the filtrate was concentrated. The residue was purified by flash chromatography (CH_2Cl_2/EtOAc, 8:1; R_f = 0.56 for CH_2Cl_2/EtOAc, 7:1) to furnish (1*S*,2*R*)-**10** (0.489 g, 64%) as a viscous oil. $[\alpha]_D^{20}$ = −1.7 (*c* = 0.9, acetone). IR (Si): $\tilde{\nu}$ = 2980, 2932, 2109, 1255, 987 cm^{-1}. ^1H NMR (250 MHz, $CDCl_3$): δ = 0.15 (s, 3 H), 0.17 (s, 3 H), 0.91 (s, 9 H), 1.30 (d, *J* = 6.2 Hz, 3 H), 1.31 (d, *J* = 6.1 Hz, 3 H), 1.32 (d, *J* = 6.3 Hz, 3 H), 1.34 (d, *J* = 6.3 Hz, 3 H), 3.89 (m, 1 H), 3.96 (dd, *J* = 2.1, 10.5 Hz, 1 H), 4.70 (m, 1 H), 4.76 (m, 1 H). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = −5.14, −4.74, 13.57, 18.31, 23.88 (d, *J* = 5.4 Hz), 23.99 (d, *J* = 3.8 Hz), 24.10 (d, *J* = 4.6 Hz), 24.35 (d, *J* = 3.1 Hz), 58.86 (d, *J* = 11.5 Hz), 70.86 (d, *J* = 7.7 Hz), 71.58 (d, *J* = 6.9 Hz), 72.57 (d, *J* = 169.0 Hz). ^{31}P NMR (162.0 MHz, $CDCl_3$): δ = 19.41. $C_{15}H_{34}N_3O_4PSi$ (379.52): calcd. C 47.47, H 9.03; found C 47.65, H 9.01.

(1*S*,2*R*)-2-Amino-1-hydroxypropylphosphonic Acid [(1*R*,2*S*)-11]: A solution of azide (1*R*,2*S*)-**9** (0.287 g, 0.756 mmol) in dry ethanol (60 mL) and concentrated HCl (1 mL) was hydrogenated over Pd/charcoal (50 mg, 10% Pd) in a Parr apparatus at a hydrogen pressure of 50 psi at room temperature for 15 h and then deprotected as described for the preparation of (1*R*,2*S*)-**8**. The residue was dried in a vacuum dessicator over KOH and purified by ion exchange chromatography (Dowex 50, H^+, elution with water until neutral, then 1 M HCO_2H). The ninhydrin positive fractions (PC, R_f = 0.67) were pooled and concentrated in vacuo to give 2-aminophosphonic acid (1*S*,2*R*)-**11** as a crystalline solid (80 mg, 68%); m.p. 262−263 °C (decomposition). $[\alpha]_D^{20}$ = +12.5 (*c* = 0.9, H_2O). IR (Nujol): $\tilde{\nu}$ = 3000 (v. br.), 1630, 1520, 1200, 1080, 920 cm^{-1}. ^1H NMR (400.1 MHz, D_2O): δ = 1.33 (d, *J* = 6.8 Hz, 3 H), 3.62 (m, 1 H), 3.83 (dd, *J* = 4.4, 11.5 Hz, 1 H). ^{13}C NMR (100.6 MHz, D_2O): δ = 13.72 (d, *J* = 3.1 Hz), 49.65 (d, *J* = 8.4 Hz), 68.94 (d, *J* = 153.7 Hz). ^{31}P NMR (162.0 MHz, D_2O): δ = 16.16. $C_3H_{10}NO_4P$ (155.09): calcd. C 23.23, H 6.50, N 9.03; found C 23.23, H 6.32, N 8.74.

Acknowledgments

We are grateful to the Fonds zur Förderung der wissenschafltichen Forschung (project no. P13017-CHE and 6537C) for financial support and Biochemie Gesellschaft (Vienna/Austria) for fosfomycin.

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[11] Compound **3**: ^1H NMR (400.1 MHz, D_2O): δ = 1.36 (d, *J* = 7.0 Hz, 3 H, CH_3), 3.55 (m, 1 H, H-2), 3.66 (dd, *J* = 5.0, 9.5 Hz, 1 H, H-1). ^{31}P NMR (162 MHz, D_2O): δ = 16.74. Compound **5**: ^1H NMR (400.1 MHz, D_2O): δ = 1.34 (d, *J* = 7.0 Hz, 3 H, CH_3), 3.63 (m, 1 H, H-2, normally obscured by other signals), 3.84 (dd, *J* = 4.0, 11.5 Hz, 1 H, H-1). ^{31}P NMR (162 MHz, D_2O): δ = 16.09.
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Received October 25, 2001
[O01518]