Studies on the biodegradation of fosfomycin: Growth of *Rhizobium huakuii* PMY1 on possible intermediates synthesised chemically

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The first step of the mineralisation of fosfomycin by *R. huakuii* PMY1 is hydrolytic ring opening with the formation of (1*R*,2*R*)-1,2-dihydroxypropylphosphonic acid. This phosphonic acid and its three stereoisomers were synthesised by chemical means and tested as their ammonium salts for mineralisation as evidenced by release of P_i. Only the (1*R*,2*R*)-isomer was degraded. A number of salts of phosphonic acids such as (±)-1,2-epoxybutyl-, (±)-1,2-dihydroxyethyl-, 2-oxopropyl-, (*S*)-2-hydroxypropyl-, (±)-1-hydroxypropyl- and (±)-1-hydroxy-2-oxopropylphosphonic acid were synthesised chemically, but none supported growth. *In vitro* C–P bond cleavage activity was however detected with the last phosphonic acid. A mechanism involving phosphite had to be discarded as it could not be used as a phosphorus source. *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. The P_i released from (1*R*,2*R*)-1,2-dihydroxypropylphosphonic acid labelled with one oxygen-18 in the PO_3H_2 group did not stay long enough in the cells to allow complete exchange of ^{18}O for ^{16}O by enzymic turnover.

Introduction

Esters of orthophosphate (P_i), characterised by four P–O bonds, are ubiquitous in the biosphere. However, the number of known natural products containing a P–C bond is limited, but steadily growing.[1] Four biologically interesting examples are given (Fig. 1). 2-Aminoethylphosphonic acid (2-AEP, **1**) was isolated first from rumen protozoa and is the most simple and widely distributed phosphonic acid.[2] Fosfomycin (**2**) containing additionally an oxirane ring is a cell-wall active antibiotic used clinically.[3] The commercial herbicide phosphinothricin (**3**) containing two P–C bonds inhibits glutamine synthetase.[4] Fosmidomycin (**4**), a potent antimalarial agent blocking the Rhomer pathway of terpenoid biosynthesis, is currently undergoing field trials.[5] Glyphosate (**5**) is a synthetic herbicide used extensively throughout the world.[6]

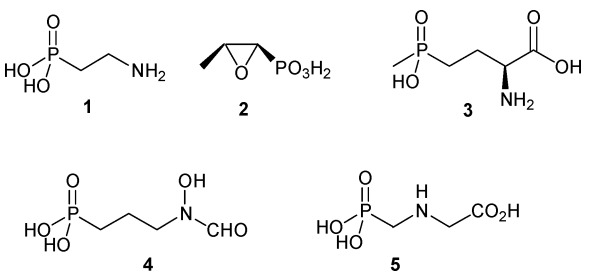

Fig. 1 Compounds containing a P–C bond.

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Although the biosynthesis of natural phosphonates and their biomineralisation (including that of man-made phosphonates) has been actively pursued, our knowledge is still fragmentary, especially with regard to their catabolism.[7] Unlike the labile P–O bond, the P–C bond is highly stable under a variety of conditions, even under forcing acidic and basic conditions.

Acquisition of adequate supplies of P_i as the preferred P source is vital for all living cells. Under conditions of P_i starvation gene systems are induced, whose products are involved in acquisition and assimilation of phosphonates. Earlier studies suggested that their breakdown is under the strict control of the *pho* regulon which is expressed only under conditions of phosphate limitation.[8] It was assumed that P–C compounds were therefore utilised only as P source, but failed to serve as a carbon (or nitrogen) source, as excess P_i released during catabolism would repress any further breakdown. The majority of these studies involved synthetic alkyl- and arylphosphonates and glyphosate, whose P–C bond is cleaved by microbial multi-enzyme systems with a broad substrate specificity ('P–C lyases') with release of P_i and a hydrocarbon.[8]

Later, P–C hydrolase enzymes and genes were found, which for the most part were not subject to control by P_i. The supplied phosphonates are used as the sole carbon and energy source for growth of the host bacterium. Phosphonoacetaldehyde hydrolase (phosphonatase), a well characterised *pho*-regulated enzyme, is part of the degradation pathway of AEP.[9] It is first transaminated (2-AEP:pyruvate aminotransferase) and then converted to acetaldehyde and P_i by phosphonatase. A *pho* independent AEP degradative operon has been identified in *Pseudomonas putida*.[7] Similarily, phosphonoacetate[10] and phosphonopyruvate[11] hydrolases cleave the respective phosphonates to give acetate and pyruvate, respectively, and P_i.

The biosynthesis of fosfomycin [*cis*-(1*R*,2*S*)-1,2-epoxypropylphosphonic acid], produced by strains of *Streptomyces*

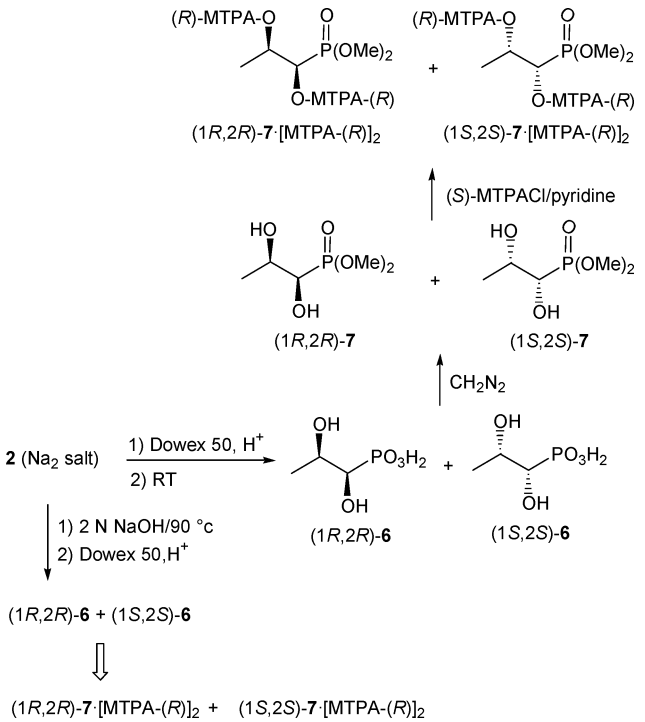
fradiae, S. wedmorensis, S. viridochromogenes and Pseudomonas syringae, has been studied extensively and features five, in-part unique steps, starting from phosphoenol pyruvate.[12] First reports showed that the cleavage of the P–C bond of fosfomycin is inducible only under conditions of phosphate limitation. Some years ago, we however reported the isolation of the bacterium *R. huakuii* PMY1 using fosfomycin as a carbon source or as a carbon and phosphorus source.[13] The mineralisation was independent of the phosphate status of the cell and resulted in essentially quantitative extracellular accumulation of organophosphonate-derived P_i. None of a series of phosphonates could replace fosfomycin. Cell extracts of *R. huakuii* PMY1 grown on fosfomycin (5 mM) as carbon, energy and phosphorus source, contained no *in vitro* P–C bond-cleaving activity either on fosfomycin or other organophosphonate substrates tested. These findings induced us to synthesise putative intermediates of the biodegradative pathway of fosfomyin and study their utilisation by *R. huakuii* PMY1.

Results and discussion

Chemical stability of oxirane ring in fosfomycin

From a chemical point of view, an oxirane ring is fairly labile under acidic and basic conditions. A nucleophile can attack fosfomycin regioselectively on either C-1 or C-2. Vidal *et al.* found that a solution of the disodium salt of fosfomycin in 0.1 N H_2SO_4, when heated at 50 °C for 24 h, furnished a diol homogeneous by paper chromatography and ^{1}H NMR spectroscopy, assuming nucleophilic attack of water at C-2.[14] Later, it was proven by the synthesis of reference samples, that the diol had indeed (*R*)-configuration at carbon atoms 1 and 2.[15] In both studies a small amount of the (1*S*,2*S*)-diol formed by attack of water at C-1 of fosfomycin might have gone undetected. To exclude that possibility, we reinvestigated the acid-catalysed ring opening of fosfomycin, and studied the base-catalysed one as well (Scheme 1). We found that its free acid, when left as an aqueous solution without added H_2SO_4 at RT for 2–3 days, was converted to diol **6** quantitatively as evidenced by NMR spectroscopy. To determine whether it was only stereoisomer (1*R*,2*R*)-**6** or in admixture with (1*S*,2*S*)-**6**, the crude diol obtained was esterified with diazomethane to give dimethyl phosphonates **7**, which were converted with (*S*)-MTPA-Cl[16] to Mosher esters (1*R*,2*R*)-**7**·[MTPA-(*R*)]$_2$ and (1*S*,2*S*)-**7**·[MTPA-(*R*)]$_2$. Similarly, an authentic sample of diol (1*S*,2*S*)-**6** (for preparation see later) was transformed into the Mosher ester (1*S*,2*S*)-**6**·[MTPA-(*R*)]$_2$. Careful inspection of their NMR spectra (^{1}H, ^{31}P) revealed that they were diastereomerically homogeneous. Therefore, acid-catalysed opening of the epoxide ring of fosfomycin furnished exclusively (>99%) diol (1*R*,2*R*)-**6**, implicating attack of water at C-2.

When a solution of fosfomycin in 2 M NaOH was left for 14 days at RT, the ratio of diol/fosfomycin was 11 : 89, indicating very slow hydrolysis of the epoxide ring. The reaction rate increased by heating the mixture at 90 °C, at which it was finished after 1 day. Part of the reaction mixture was converted to the free acid using Dowex 50, H^+, followed by esterification to give Mosher esters of dimethyl phosphonates **7** as before. This time, the NMR spectra revealed that it was a mixture, derived from diols (1*R*,2*R*)- and (1*S*,2*S*)-**6** in a ratio of 10 : 1. Base-catalysed ring opening of fosfomycin is less regioselective than the acid-catalysed one.



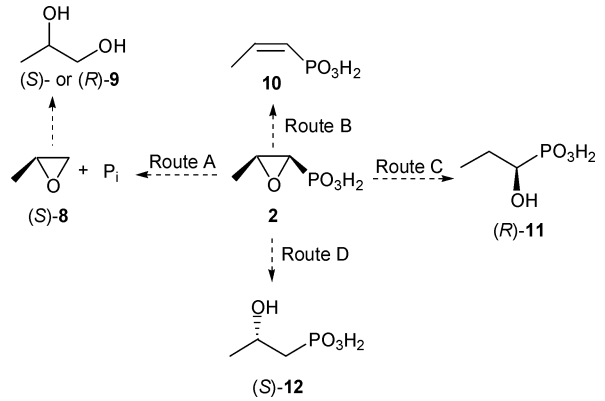
Scheme 1 Acid and base-catalysed opening of oxirane ring in fosfomycin.

Compared to H_2O and OH^-, ammonia[17] attacks the epoxide ring less regioselectively (C-2 : C-1 = 2.5 : 1), CF_3CO_2H, HCl and HBr attack[18] at C-2. The mode of action of fosfomycin and the mode of resistance to it are based on the alkylation of the sulfur atoms of cysteins of specific proteins by fosfomycin, whereby inversion of configuration is caused at C-2[19] on oxirane ring opening in the former case and at C-1[20] in the latter.

Chemical synthesis of possible intermediates and their use in degradative studies

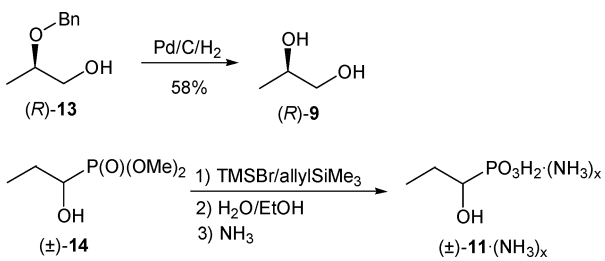
With the aim of unravelling the stepwise mineralisation of fosfomycin by *R. huakuii* PMY1, we decided to prepare putative intermediates of the catabolic pathway and test them as phosphorus, carbon and energy sources for this bacterium. Additionally for those organophosphonate substrates under test, to negate the possibility of their lack of transport into the cell, studies were also performed on cell-free extracts prepared from fosfomycin grown cells of *R. huakuii* PMY1. As virtually nothing was known about the biodegradation of natural organophosphonates when we started this work, we tested even unlikely intermediates as outlined in Scheme 2.

Direct enzymatic cleavage of the P–C bond of fosfomycin by a specific P–C lyase could yield P_i and epoxide (*S*)-**8**, which might be hydrolysed by an epoxide hydrolase to give diol (*S*)- or (*R*)-**9**, depending on which carbon atom inversion was effected (Route A). Deoxygenation of fosfomycin could give *cis*-1-propenylphosphonic acid (**10**) (Route B) and reductive ring opening either hydroxypropylphosphonic acid (*R*)-**11** or (*S*)-**12** (Routes C and D).



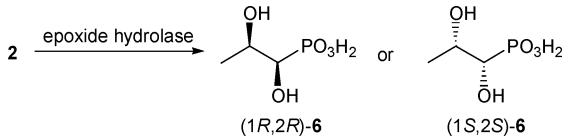
Scheme 2 Putative intermediates of biodegradation of fosfomycin.

Therefore, we had to get the putative intermediates **9**, of which we wanted to test the racemic and (*R*)-configured form, **10**, and the racemates of **11** and **12**. However, epoxide (*S*)-**8** is very volatile (bp 35 °C) and was not considered a suitable compound for testing. (±)-1,2-Propanediol (**9**) is commercially available, the (*R*)-enantiomer was obtained by deblocking the corresponding benzyl ether[21] (*R*)-**13** (Scheme 3). Phosphonic acids **10**[22] and (±)-**12**[12c] left as their cyclohexylammonium salts from previous studies on the biosynthesis of fosfomycin were converted to the ammonium salts (Dowex 50,H^+, then neutralisation with ammonia). The ammonium salt of (±)-**11** was easily accessible by deblocking hydroxyphosphonate (±)-**14**[23] using mild conditions[15] (Me_3SiBr/allylSiMe_3), followed by neutralisation of the free acid with ammonia. This ammonium salt and others obtained by lyophilisation as fluffy powders were homogeneous by NMR and contained varying amounts of nitrogen and water as found by microanalysis. They were normally used directly for the degradative studies, assuming a 95% yield for the preparation of the ammonium salts. They could be purified by ion exchange chromatography (Dowex 1, HCO_3^- form), if necessary. Unfortunately, none of these putative intermediates was used by *R. huakuii* PMY1 as carbon and phosphorus sources. Additionally no *in vitro* C–P bond cleavage activity was detected. We therefore envisoned formal opening of the epoxide ring by water as an alternative first step in the mineralisation of fosfomycin (Scheme 4).



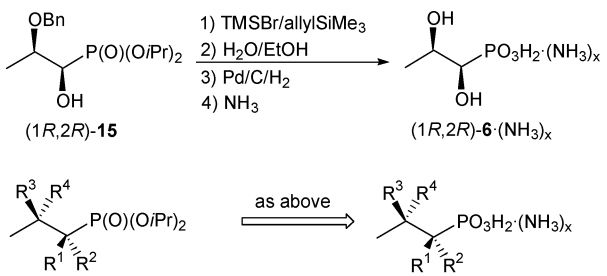
Scheme 3 Synthesis of putative intermediates **9** and **11** of biodegradation of fosfomycin.

Depending on the regioselectivity of the enzyme, diol **6** could have either (1*R*,2*R*)- or (1*S*,2*S*)-configuration, if water attacked at C-2 or C-1 of fosfomycin, respectively. The former isomer was prepared by two methods. The first one was based on the protected diol (1*R*,2*R*)-**15** prepared by base-catalysed addition of diisopropyl phosphite to (*R*)-*O*-benzyllactaldehyde.[15] The isopropyl



Scheme 4 Conversion of fosfomycin by an epoxide hydrolase to diol (1*R*,2*R*)- or (1*S*,2*S*)-**6**.

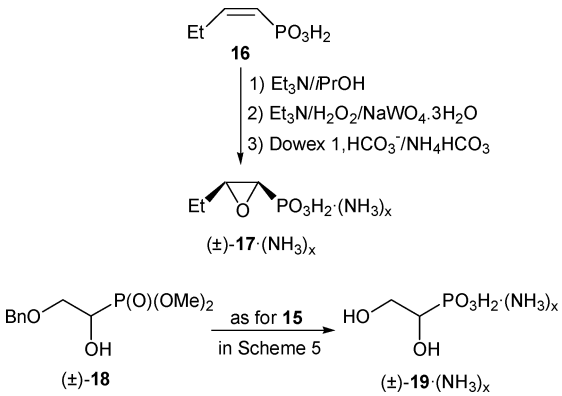
groups were removed with TMSBr/allylsilane and the benzyl group by catalytic hydrogenation (Scheme 5).[15] The second and more convenient method was to keep an aqueous solution of the free acid of fosfomycin at RT (see Scheme 1), neutralise it with NH_3 and finally lyophilise it. When this salt was added to growth medium (5 mM) of *R. huakuii* PMY1, it was used as a carbon and phosphorus source with concomitant release of P_i (4.1 mM) into the growth medium in virtually quantitiative yield. To test the degradation of the other stereoisomers of (1*R*,2*R*)-**6** as well, their ammonium salts were prepared similarly. Surprisingly, none supported growth as evidenced by the lack of release of P_i. These findings in combination with the lack of growth of *R. huakuii* on (*R*)-1,2-propanediol [(*R*)-**9**] show that (1) the first step of the biodegradation of fosfomycin is the stereospecific epoxide ring opening at C-2 by a putative epoxide hydrolase and (2) that the following step is not cleavage of the P–C bond. If that were to occur, P_i and (*R*)-**9** would result. The metabolism of the latter would be a prerequisite for its use as an energy and carbon source. However, it does not take place. Moreover no *in vitro* cleavage of the P–C bond of (1*R*,2*R*)-**6** could be detected. Additionally, we fed the ammonium salts of phosphonic acids (±)-**17** and (±)-**19**, homologues of fosfomycin and 1,2-dihydroxypropylphosphonic acid, respectively, to *R. huakuii.* The former was prepared by epoxidation of the triethylammonium salt of *cis*-1-butenylphosphonic acid (**16**) with $H_2O_2/Na_2WO_4{\cdot}H_2O$ and isolation as ammonium salt by ion exchange chromatography,[12d] the latter from hydroxyphosphonate (±)-**18**[24] (Scheme 6). None supported growth or were degraded in cell-extract preparations.



	R^1	R^2	R^3	R^4		R^1	R^2	R^3	R^4
(1*S*,2*S*)-**15**	H	OH	H	OBn	(1*S*,2*S*)-**6**·$(NH_3)_x$	H	OH	H	OH
(1*R*,2*S*)-**15**	OH	H	H	OBn	(1*R*,2*S*)-**6**·$(NH_3)_x$	OH	H	H	OH
(1*S*,2*R*)-**15**	H	OH	OBn	H	(1*S*,2*R*)-**6**·$(NH_3)_x$	H	OH	OH	H

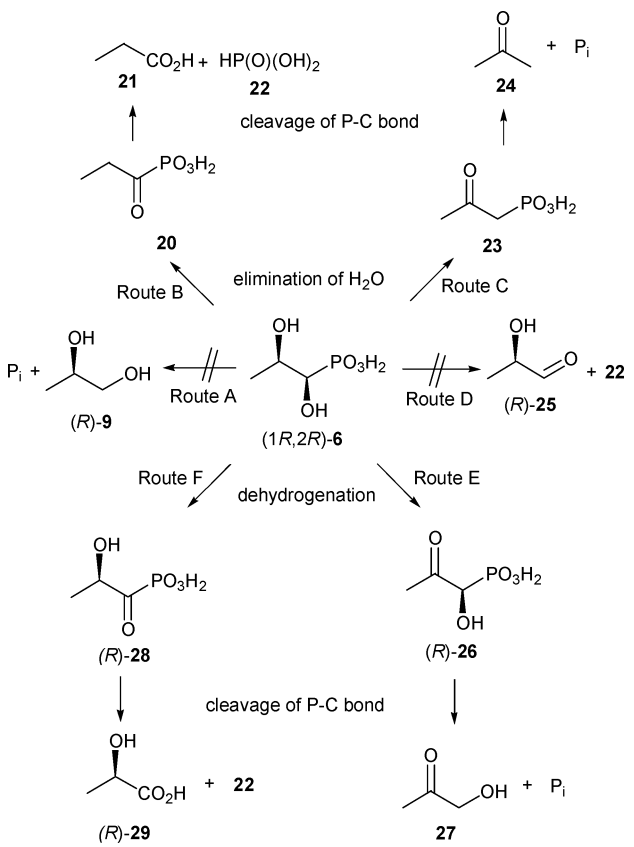
Scheme 5 Conversion of protected stereoisomeric hydroxyphosphonates **15** to the ammonium salts of the corresponding acids **6**.

The next step on the biodegradative pathway of 1,2-dihydroxypropylphosphonic acid (1*R*,2*R*)-**6** cannot be the cleavage of the P–C bond by a P–C lyase as the resulting propanediol [(*R*)-**9**] was not metabolised as already found. Evidently, (1*R*,2*R*)-**6** is modified in the side chain before the P–C bond



Scheme 6 Preparation of ammonium salts of epoxy- and dihydroxyphosphonic acids (±)-**17**·and (±)-**19**.

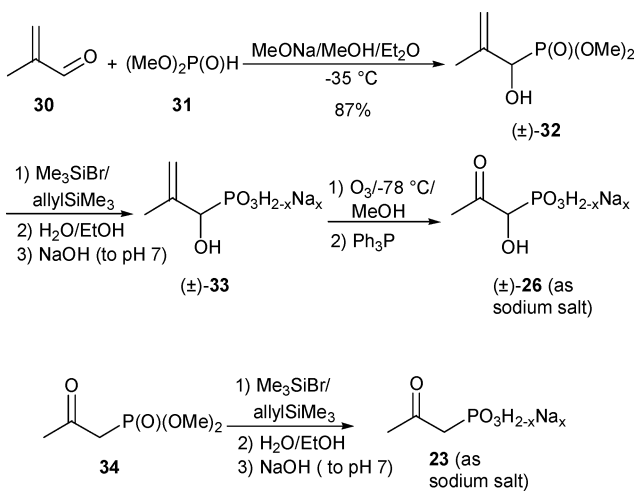
is split (Scheme 7). Enzymatic elimination of water from the diol could give either 1-oxopropylphosphonic acid (**20**) or 2-oxopropylphosphonic acid (**23**), depending on the hydroxyl group which is removed. Dehydrogenation however, could yield isomeric hydroxy-oxopropylphosphonic acids (*R*)-**26** or (*R*)-**28**. Whereas the P–C bond in alkyl- and arylphosphonic acid esters are extremely stable chemically, those in α-hydroxyalkyl- and α-oxoalkylphosphonic acid esters are fairly labile. The former are base labile and are split to give dialkyl phosphite and aldehydes or ketones, from which they are formed easily (Pudovik[25] and retro-Abramov reaction[26]). α-Oxoalkylphosphonic acid esters are even more labile and are hydrolysed to dialkyl phosphites and



Scheme 7 Putative intermediates for degradation of fosfomycin beyond (1*R*,2*R*)-1,2-dihydroxypropylphosphonic acid.

carboxylic acids.[27] However, the corresponding salts show increased stability compared to the free acids. Possibly, an enzyme with a new mode of action, could hydrolyse α-hydroxyphosphonate (1*R*,2*R*)-**6** to lactaldehyde [(*R*)-**25**] and phosphite (**22**, Route D) or α-oxophosphonates **20** and (*R*)-**28** to propionic acid [**21**, Route B] and lactic acid [(*R*)-**29**, Route F], respectively, and phosphite. Naturally, cleavage of the P–C bonds in these latter substrates as well as in **23** and (*R*)-**26** by a novel P–C lyase, whereby P_i and aldehydes and acetone (**24**) and hydroxyacetone (**27**), respectively, are formed, is also possible. Metcalf and Wolfe[28] found that phosphite-oxidising organisms may in fact be quite common and Vrtis *et al.*[29] studied the mechanism of a phosphite dehydrogenase.

However phosphite could not be used as a phosphorus source by *R. huakuii* PMY1 and no phosphite oxidase activity (using both phosphorous acid and sodium phosphite as substrates) could be detected in PMY1 cell-free-extracts. Some of the mechanisms outlined in Scheme 7 must therefore be dismissed (Routes B, D, and F). Furthermore, *R. huakuii* grew very well on hydroxyacetone (**27**), (*R*)- and (*S*)-lactic acid [(*R*)- and (*S*)-**29**], but less well on propionic acid and not on acetone. Thus route E was the only one left for the degradation of (1*R*,2*R*)-**6**. We decided to synthesise **26**, in the first place in its racemic form, and its analogue **23** without the hydroxyl group (Scheme 8). Base-catalysed addition of dimethyl phosphite (**31**) to metacrolein (**30**) furnished α-hydroxyphosphonate (±)-**32** in 87% yield. It was deblocked as usual and converted to the sodium salt, which was ozonolysed to give the desired 2-oxopropylphosphonic acid (±)-**23** as sodium salt. The analogue without the hydroxyl group was produced by deblocking 2-oxopropylphosphonate **34** and neutralising the acid with NaOH. Ammonia was replaced in these two cases here by NaOH, as ammonia might react with the carbonyl groups and give side products.

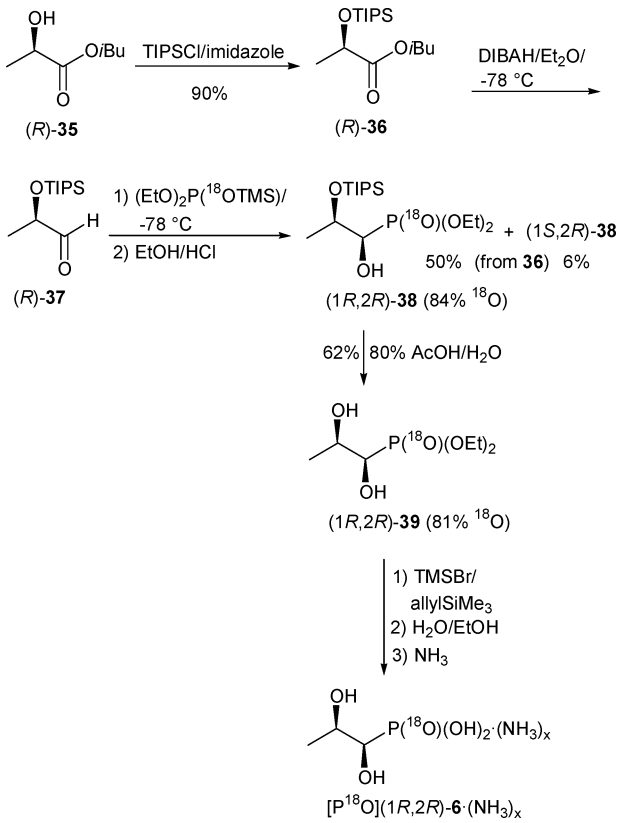


Scheme 8 Synthesis of **2**-oxopropylphosphonic acids **23** and (±)-**26** (as sodium salts).

The sodium salt of **23** was not used as carbon and phosphorus source by *R. huakuii* PMY1 as anticipated. Surprisingly, the putative intermediate (±)-**26** was not used either. To account for this unexpected finding in combination with the fact that hydroxyacetone is degraded, we have to assume either that phosphonic acid (±)-**26** does not enter the cells or that (*S*)-**26**

acts as a growth inhibitor [since it is most likely to be (*R*)-**26** formed *in vivo* from the (1*R*,2*R*)-**6** precursor]. Yet *in vitro* C–P bond cleavage activity using (±)-**26** as a substrate was detectable. Control assays containing no cell-extract or no substrate were performed to confirm this.

Finally, we addressed the length of stay of P_i formed by the cleavage of the P–C bond in the the cells using (1*R*,2*R*)-**6** with an oxygen-18 labelled phosphonate group (Scheme 9). Thus, isobutyl lactate [(*R*)-**35**] was TIPS-protected[30] and reduced with DIBAH at −78 °C to give lactaldehyde [(*R*)-**37**], to which ^{18}O-labelled diethyl trimethylsilyl phosphite was added at low temperature diastereoselectively.[31] The labelled phosphite was obtained by silylation[32] of ^{18}O-labelled diethyl phosphite[33] derived from triethyl phosphite and $H_2{}^{18}O$. The reaction mixture was treated with HCl in EtOH to remove the trimethylsilyl group from the α-hydroxy group to furnish a mixture of diastereomers separable by flash chromatography [(1*R*,2*R*)-**38**-(1*S*,2*R*)-**38**, 89 : 11]. Deblocking of the major diastereomer (84% oxygen-18 labelled) yielded the ammonium salt of the ^{18}O-labelled (1*R*,2*R*)-**6**. The ^{18}O-labelled phosphonic acid was used by *R. huakuii* PMY1 as a carbon and phosphorus source. After release of P_i (4 mmol) from 5 mM phosphonate, cells were removed by centrifugation and the supernatant was freeze-dried. The residue was found to contain inorganic phosphate (0.72% w/w) and the ratio of $PO_4^{3-}/PO_3{}^{18}O^{3-}$ was 95.6 : 4.4 as determined by LC-MS. Evidently, the inorganic phosphate does not stay long enough in the cells after release from the labelled precursor to allow complete exchange of ^{18}O for ^{16}O by enzymic turnover.



Scheme 9 Preparation of ammonium salt of (1*R*,2*R*)-**6** ^{18}O-labelled in the phosphonate group.

Conclusions

In summary we have shown that fosfomycin is stereospecifically opened under acidic conditions to give the (1*R*,2*R*)-1,2-dihydroxypropylphosphonic acid exclusively and under basic conditions in admixture with 9% of the (1*S*,2*S*)-enantiomer. The first step of the mineralisation of fosfomycin by *R. huakuii* PMY1 is the hydrolytic ring opening with the formation of (1*R*,2*R*)-1,2-dihydroxypropylphosphonic acid. Of the four stereoisomeric 1,2-dihydroxypropylphosphonic acids prepared chemically as ammonium salts only the (1*R*,2*R*)-isomer was mineralised as evidenced by release of P_i and growth. A number of the phosphonic acids considered as putative intermediates on the biodegradative pathway of fosfomycin were synthesised chemically and tested for growth and release of P_i. None supported growth; *in vitro* C–P bond cleavage activity was however detected with racemic 1-hydroxy-2-oxopropylphosphonic acid. A mechanism involving phosphite had to be dismissed as it could not be used as phosphorus source. Hydroxyacetone, a putative intermediate of the catabolic pathway, supported growth.

Experimental

General experimental

^1H, ^{13}C and ^{31}P NMR spectra were recorded in $CDCl_3$ and D_2O at 300 K on a Bruker Avance DRX 400 at 400.13, 100.61 and 161.98 MHz, respectively. Chemical shifts were referenced to residual $CHCl_3$ (δ_H 7.24) or HDO (δ_H 4.80) and $CDCl_3$ (δ_C 77.00) and external H_3PO_4 (85%). Chemical shifts are given in δ in ppm and *J* values in Hz. The FD mass spectra were recorded on a Finnigan MAT 900S at 30 °C. IR spectra were run on a Perkin-Elmer 1600 FT-IR spectrometer; liquid samples were measured as films on a silicon disc.[34] Optical rotations were measured at 20 °C on a Perkin-Elmer 351 polarimeter in a 1 dm cell. TLC was carried out on 0.25 mm thick Merck plates, silica gel 60 F_{254}. Flash (column) chromatography was performed with Merck silica gel 60 (230–400 mesh). Spots were visualised by UV and/or dipping the plate into a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (23.0 g) and of $Ce(SO_4)_2{\cdot}4H_2O$ (1.0 g) in 10% aqueous H_2SO_4 (500 mL), followed by heating with a heat gun. Phosphonic acids eluted from Dowex 1, HCO_3^- were also detected by TLC (*i*PrOH–H_2O–conc. NH_3 6 : 3 : 1, then visualising). The following ion exchange resins were used: Dowex 1 × 8, HCO_3^- (100–200 mesh) Dowex 50 × 8, H^+ (50–100 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 aluminium oxide 90 active neutral (0.063–0.200 mm, activity I) and stored over molecular sieves (3 Å 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 were uncorrected.

Determination of isotopic composition of inorganic phosphate by LC-MS: HPLC HP 1100 with UV/Vis Diode Array Detector, equipped with a vacuum degasser and quaternary pump and a HP 1050 Autosampler (all Hewlett Packard, Palo Alto, CA, USA); Mass spectrometry: quadrupole system HP 5989 B (Hewlett Packard) with Atmospheric Pressure Ionisation Interface HP

59987 A (Hewlett Packard) equipped with an RF-only hexapole (Analytica of Branford, Branford, CT, USA).

It was assumed that the conversion of phosphonates to the respective ammonium or sodium salts was effected in 95% yield. Ammonium salts of hydroxyphosphonic acids could be purified by ion exchange chromatography by analogy to the conversion of 1,2-dihydroxypropylphosphonic acid × 1.5 cyclohexylamine to the ammonium salt given below, if necessary.

Conversion of 1,2-dihydroxypropylphosphonic acid × 1.5 cyclohexylamine (prepared from the disodium salt of fosfomycin) to ammonium salt. The salt (0.300 g, 0.984 mmol) was dissolved in water and applied to a column filled with Dowex 1 × 8, HCO_3^- (75 mL, i.d. 2 cm × 23 cm) and eluted with aqueous NH_4HCO_3 (0.1 M, 350 mL, then 0.25 M; fractions of 25 mL). The phosphonic acid was eluted in fractions 19–25 (TLC, R_f 0.42). Fractions containing product were pooled, concentrated under reduced pressure to a small volume and lyophilised to give the ammonium salt as a white, fluffy product (0.176 g).

(1*R*,2*R*)-Dihydroxypropylphosphonic acid, ammonium salt [(1*R*,2*R*)-6·$(NH_3)_x$], prepared from fosfomycin. Fosfomycin (1.32 g of the disodium salt, 7.25 mmol, dissolved in 5 mL of water) was passed down a column of Dowex 50 × 8, H^+ (30 mL). It was eluted with water until neutral. The solution (about 50 mL) was left at RT for 48 h and then basified with ammonia (25%, 2 mL). Lyophilisation gave the ammonium salt (1.470 g) as a hygroscopic fluffy powder. Lyophilisation of the aqueous solution without prior addition of ammonia gave the oily free acid. Ammonium salt: δ_H (400.1 MHz; D_2O) 4.06 (1 H, m, PCCH), 3.44 (1H, dd, *J* 9.6, 4.3, PCH), 1.26 (3 H, d, *J* 6.6, CH_3); δ_C (100.6 MHz, D_2O) 73.2 (PCH, d, *J* 145.3), 68.3 (CH, d, *J* 3.1), 18.9 (CH_3, d, *J* 7.7); δ_P (162 MHz, D_2O) 17.4.

Synthesis of all four stereoisomeric 1,2-dihydroxypropylphosphonic acids, ammonium salts [(1*R*,2*R*)-, (1*S*,2*S*)-, (1*R*,2*S*)- and (1*S*,2*R*)-6·$(NH_3)_x$], from protected precursors 15. They were prepared by deblocking the corresponding diisopropyl 2-benzyloxy-1-hydroxypropylphosphonates **15**, which were obtained by analogy to the compounds deuterated at C-1, but starting from nondeuterated (*R*)- and (*S*)-2-benzyloxypropanal.[15] Diisopropyl phosphite was added to the aldehyde in THF, catalysed by DBU (–78 °C to room temperature). The ratio of the diastereomeric phosphonates was 1 : 1 (^{1}H NMR, C_6D_6). Homogeneous samples were obtained by flash chromatography (CH_2Cl_2-EtOAc 5 : 1, TLC for 3 : 1, (1*R*,2*R*)-**15**: R_f 0.38; (1*S*,2*R*)-**15**: 0.31) and the combined yields were 60–70%.

(1*R*,2*R*)-15. Colourless needles (hexanes), mp 83 °C (lit.[15] for C-1 deuterated compound: 84–86 °C); $[\alpha]_D^{20}$ –24.2 (*c* 2.0 in CH_2Cl_2) {lit.[15] for C-1 deuterated compound: $[\alpha]_D^{20}$ –22.8 (*c* 1.63 in CH_2Cl_2)} and (1*S*,2*R*)-**15**, colourless oil, $[\alpha]_D^{20}$ –6.3 (*c* 2.0 in CH_2Cl_2) {lit.[15] for C-1 deuterated compound: $[\alpha]_D^{20}$ –5.8 (*c* 2.08 in CH_2Cl_2)} were obtained from (*R*)-2-benzyloxypropanal.

(1*S*,2*S*)-15. Colourless needles (hexanes), mp 83 °C, $[\alpha]_D^{20}$ +23.5 (*c* 2.18 in CH_2Cl_2) and (1*S*,2*R*)-**15**, colourless oil, $[\alpha]_D^{20}$ +6.5 (*c* 2.0 in CH_2Cl_2), were obtained from (*S*)-2-benzyloxypropanal.

The ^{1}H and ^{13}C NMR spectra of (1*R*,2*R*)- and (1*S*,2*S*)-**15** and of (1*S*,2*R*)- and (1*R*,2*S*)-**15**, respectively, were identical. Their ^{1}H NMR spectra were identical to those of the corresponding racemates.

(1*R*,2*R*)-15. δ_C (100.6 MHz; $CDCl_3$) 138.0, 128.4 (2 C), 128.0 (2 C), 127.7, 74.3 (d, *J* 3.8), 72.4 (d, *J* 162.9), 71.4 (d, *J* 6.9), 71.4, 70.8 (d, *J* 6.9), 24.2 (d, *J* 3.1), 24.04 (d, *J* 4.6), 24.0 (d, *J* 4.6), 23.8 (d, *J* 5.4), 16.7 (d, *J* 7.7).

(1*S*,2*R*)-15. δ_C (100.6 MHz; $CDCl_3$) 138.2, 128.3 (2 C), 127.8 (2 C), 127.5, 74.9 (d, *J* 6.1), 71.2 (d, *J* 6.9), 71.1 (d, *J* 6.9), 70.7, 70.6 (d, *J* 159.9), 24.1 (d, *J* 3.8), 24.0 (d, *J* 3.8), 23.8 (d, *J* 4.6), 23.8 (d, *J* 5.4), 16.7 (d, *J* 3.8).

Deblocking of phosphonates 15. The four diisopropyl phosphonates were deblocked according to a literature procedure[15] except that CCl_4 was replaced by dry 1,2-dichloroethane (molar ratio of **15**-allylTMS-TMSBr 1 : 1.5 : 5; 17 h at 50 °C). After hydrogenolytic removal (Pd/C/H_2) of the benzyl group in dry EtOH and removal of the catalyst, ammonia (25%) was added and the solution was concentrated. The residue was dissolved in water and lyophilisation gave fluffy or gum-like ammonium salts. If there was still EtOH present in the salt (by ^{1}H NMR in D_2O), it was dissolved again in water and lyophilised. It was assumed that the conversion of the phosphonates to the respective ammonium salts was effected in 95% yields. These salts were used directly for the degradation studies.

The spectroscopic data (^{1}H, ^{13}C and ^{31}P NMR; D_2O) of the ammonium salts of (1*R*,2*R*)- and (1*S*,2*S*)-**6** were identical to the one derived from fosfomycin. The spectroscopic data of (1*R*,2*S*)- and (1*S*,2*R*)-**6** were identical; δ_H (400.1 MHz; D_2O) 4.06 (1 H, m, PCCH), 3.62 (1 H, dd, *J* 10.1, 5.3, PCH), 1.28 (3 H, d, *J* 6.6, CH_3); δ_C (100.6 MHz; D_2O) 73.7 (d, *J* 146.9), 68.5 (d, *J* 6.9), 17.6 (d, *J* 5.4); δ_P (162 MHz; D_2O) 17.6.

Base-catalysed ring opening of fosfomycin. A solution of fosfomycin (Na_2 salt, 0.500 g) in 2 M NaOH (10 mL) was heated at 90 °C for 24 h. After 17 h a sample (0.25 mL) was withdrawn, neutralised with solid CO_2 and lyophilised. The residue was dissolved in D_2O to record a ^{1}H and ^{31}P NMR spectrum (besides diol there was still 3% of fosfomycin present). The cold solution was diluted with water and applied to a column of Dowex 50 × 8, H^+. Washing with water gave an eluate, which was basified with ammonia (25%) and lyophilised to give the ammonium salt containing a small amount of an impurity.

Preparation of Mosher ester {7·[MTPA-(*R*)]$_2$}. Dimethyl 1,2-dihydroxypropylphosphonate (**7**) was derived from fosfomycin (by acid- or base-catalysed ring opening) and (1*S*,2*S*)-**6**. A sample (up to 50 mg) of 1,2-dihydroxypropylphosphonic acid derived from fosfomycin (by acid- or base-catalysed ring opening) or (1*S*,2*S*)-**6** was esterified in dry MeOH with a distilled etheral solution of diazomethane at 0 °C. Concentration of the solution yielded a mixture of dimethyl 1,2-dihydroxypropylphosphonate and its isomeric monomethyl ethers (10 : 1 : 1, by ^{1}H and ^{31}P NMR). A portion of the mixture (15 mg) was esterified at RT in dry CH_2Cl_2 (1 mL) containing dry pyridine (5 drops) and (*S*)-MTPACl (0.5 mL of a 0.5 M solution in dry CH_2Cl_2). After 18 h the solution was concentrated under reduced pressure and water and CH_2Cl_2 were added to the residue. The organic phase was washed with 2 M HCl, a saturated aqueous solution of $NaHCO_3$, dried (Na_2SO_4) and concentrated under reduced

pressure to give an oil. Part of it was investigated directly by ^{1}H and ^{31}P NMR spectroscopy, the remaining portion after flash chromatography (hexanes-EtOAc 1 : 1, R_f 0.13 for 1 : 2).

(1*R*,2*R*)-7·[MTPA-(*R*)]$_2$. δ_H (400.1 MHz; $CDCl_3$) 7.56–7.32 (10 H, m), 5.58–5.60 (1 H, m), 5.52 (1 H, dd, *J* 12.9, 3.3, PCH), 3.63 (3 H, d, *J* 10.9, OCH_3), 3.48 (3 H, d, *J* 10.9, OCH_3), 3.47 (3 H, q, *J* ~1, OCH_3), 3.44 (3 H, q, *J* ~1, OCH_3), 1.34 (3 H, dd, *J* 6.6, 1.3, CH_3); δ_P (162 MHz; $CDCl_3$) 17.9.

(1*S*,2*S*)-7·[MTPA-(*R*)]$_2$. δ_H (400.1 MHz; $CDCl_3$) 7.60–7.31 10 H, m), 5.60–5.49 (2 H, m), 5.55 (3 H, d, *J* 10.9, CH_3), 3.48 (3 H, q, *J* ~1, OCH_3), 3.44 (3 H, q, *J* 10.9, OCH_3), 3.43 (3 H, q, *J* ~1, CH_3), 1.32 (3 H, d, *J* 6.6, CH_3); δ_P (162 MHz; $CDCl_3$) 18.2.

(*R*)-(−)-1,2-Propanediol [(*R*)-9]. (*R*)-(−)-2-Benzyloxy-propanol[21] [3.00 g, 18.05 mmol; α_D^{20} −28.0 (neat), obtained by reduction of (*R*)-(+)-isobutyl *O*-benzyllactate with $LiAlH_4$] was hydrogenated over Pd/C (0.20 g, 10%) in dry ethanol (60 mL) containing 2 drops of conc. HCl in a Parr apparatus at 3.4 bar for 3 h at RT. The catalyst was removed and the filtrate was concentrated under reduced pressure. The crude product was bulb to bulb distilled (80 °C/10 mm) (lit.[35] 92 °C/14 mm) to give diol (*R*)-**9** (0.79 g, 58%) as a colourless oil; $[\alpha]_D^{20}$ −24.8 (*c* 1.02 in $CHCl_3$) {lit.[35] $[(\alpha)]_D^{24.4}$ −28.6 (in $CHCl_3$)}; δ_H (400.1 MHz; $CDCl_3$) 3.86 (1 H, qdd, *J* 7.9, 6.4, 3.0, CHO), 3.57 (1 H, dd, *J* 11.3, 3.0, one of CH_2O), 3.35 (1 H, dd, *J* 11.3, 7.9, one of CH_2O), 3.13 (2 H, s, 2 × OH), 1.13 (3 H, d, *J* 6.4, CH_3).

(±)-1-Hydroxypropylphosphonic acid, ammonium salt [(±)-11·(NH$_3$)$_x$]. A solution of (±)-dimethyl 1-hydroxypropylphosphonate (0.325 g, 1.93 mmol), allyltrimethylsilane (0.332 g, 0.46 mL, 2.90 mmol) and TMSBr (1.48 g, 1.27 mL, 9.65 mmol) in dry 1,2-dichloroethane (8 mL) was heated at 50 °C under argon for 2 h. After cooling, volatile components were removed under reduced pressure (0.5 mm). The residue was dissolved in dry 1,2-dichloroethane (5 mL) and again concentrated under reduced pressure. The residue was then dissolved in a mixture of ethanol and water (10 mL, 1 : 1) and after 15 min concentrated at reduced pressure and dissolved in water (5 mL)/conc. ammonia (0.5 mL). Lyophilisation gave the ammonium salt of (±)-**11** as a white powder (0.312 g); δ_H (400.1 MHz; D_2O) 3.61 (1 H, ddd, *J* 10.1, 6.8, 3.3, PCH), 1.83 (1 H, m), 1.60 (1 H, m), 1.04 (3 H, t, *J* 7.4, CH_3); δ_C (100.6 MHz, D_2O) 71.0 (d, *J* 156.8), 25.1 (d, *J* 1.5), 10.7 (d, *J* 13.7); δ_P (162 MHz; D_2O) 21.7.

***cis*-(±)-1,2-Epoxybutylphosphonic acid, ammonium salt [(±)-17·(NH$_3$)$_x$].** The crude product obtained by epoxidation[12d] of *cis*-1-butenylphosphonic acid (as triethylammonium salt) was purified by ion exchange chromatography (Dowex 1 × 8, HCO_3^-) by eluting first with 0.1 M NH_4HCO_3, then 0.25 M. The fractions containing the epoxyphosphonic acid (TLC, R_f 0.62) were pooled and lyophilised to give (±)-**17**·$(NH_3)_x$ as a white foam; δ_H (400.1 MHz; D_2O) 3.00 (1 H, m, PCCH), 2.73 (1 H, dd, *J* 18.8, 4.8, PCH), 1.69 (2 H, m), 0.90 (3 H, t, *J* 7.5, CH_3); δ_P (162 MHz; D_2O) 11.5.

(±)-1,2-Dihydroxyethylphosphonic acid, ammonium salt [(±)-19·(NH$_3$)$_x$]. (±)-Dimethyl 2-benzyloxy-1-hydroxethyl-phosphonate **18**[24] (0.781 g, 3 mmol) was deblocked according to the procedure used for phosphonates **15**, except that demethylation was performed at RT for 2 h. The free phosphonic acid in EtOH after removal of the benzyl group was neutralised with ammonia (25%, 0.5 mL). The solution was concentrated under reduced pressure. The residue was dissolved twice in water (2 × 20 mL) and lyophilised to give the ammonium salt of (±)-**19** as a viscous oil (0.600 g); δ_H (400.1 MHz; D_2O) 3.91 (1 H, ddd, *J* 11.6, 4.6, 2.4, PCH), 3.83–3.65 (2 H, m, CH_2); δ_c (100.6 MHz; D_2O) 71.4 (d, *J* 147.6), 63.5 (d, *J* 9.2); δ_P (162 MHz; D_2O) 16.8.

2-Oxopropylphosphonic acid (23), sodium salt. A solution of dimethyl 2-oxopropylphosphonate (0.347 g, 2.09 mmol), allyltrimethylsilane (0.358 g, 0.50 mL, 3.14 mmol) and TMSBr (1.60 g, 1.38 mL, 10.45 mmol) in dry 1,2-dichloroethane (8 mL) was heated at 50 °C for 2 h under argon. After cooling, volatile components were removed under reduced pressure (0.5 mbar). The residue was dissolved in dry 1,2-dichloroethane (5 mL) and again concentrated under reduced pressure. The residue was dissolved in a mixture of ethanol and water (10 mL, 1 : 1) and concentrated after 15 min at reduced pressure and dissolved in water (5 mL). After bringing the pH to 7.0 (1 M NaOH) the product was lyophilised to give the sodium salt of **23** as a white powder (0.353 g); δ_H (400.1 MHz; D_2O) 3.017 (2 H, d, *J* 21.2, PCH_2), 2.35 (3 H, s, CH_3); δ_C (100.6 MHz; D_2O) 212.016 (d, *J* 4.6), 48.136 (d, *J* 107.1), 30.9; δ_P (162 MHz; D_2O) 12.1. The hydrogen atoms of the PCH_2CO group were exchanged for deuterium. After leaving the NMR probe for 5.5 h at room temperature, the ratio of PCH_2-PCHD-PCD_2 was 0.36 : 0.48 : 0.16; δ_H (400.1 MHz; D_2O) 3.000 (br. d, *J* 21.2, PCHD); δ_C (100.6 MHz; D_2O) 212.061 (d, *J* 4.6, CHD*C*O), 47.840 (td, *J* 107.1, 19.1, PCHD).

(±)-Dimethyl 1-hydroxy-2-methyl-2-propenylphosphonate [(±)-32]. A saturated solution of MeONa in MeOH (0.1 mL) was added to a stirred solution of 2-methyl-2-propenal (0.70 g, 10 mmol) and dimethyl phosphite (1.10 g, 10 mmol) in dry Et_2O (20 mL) at −35 °C under argon. After stirring for 25 min at −35 °C, conc. H_2SO_4 (7 drops) was added and the solvent was removed (−35 °C/0.5 mm). The residue was purified by flash column chromatography (EtOAc-$CHCl_3$ 9 : 1, R_f 0.28) to yield hydroxyphosphonate (±)-**32** (1.57 g, 87%) as a colourless oil (Found: C, 39.9, H, 7.5. $C_6H_{13}O_4P$ requires C, 40.0, H, 7.3%); ν_{max}/NaCl, film/cm^{-1} 3299, 2958, 1649, 1236, 1033; δ_H (400.1 MHz; $CDCl_3$) 5.17 (1 H, dq, *J* 4.9, 1.0, CH=), 5.05 (1 H, dq, *J* 2.5, 1.0, CH=), 4.43 (1 H, dd, *J* 12.8, 5.9, PCH), 4.01 (1 H, dd, *J* 9.4, 5.9, OH), 3.788 and 3.785 (2 × 3 H, 2 d, *J* 10.8, 2 × OCH_3), 1.87 (3 H, dt, *J* 3.0, 1.0, CH_3); δ_C (100.6 MHz; $CDCl_3$) 140.8 (d, *J* 3.8), 113.9 (d, *J* 11.1), 71.8 (d, *J* 157.1), 53.7 (d, *J* 6.9), 53.6 (d, *J* 7.1), 19.5 (d, *J* 2.2).

(±)-1-Hydroxy-2-oxopropylphosphonic acid [(±)-26], sodium salt. A solution of hydroxyphosphonate (±)-**31** (0.62 g, 3.4 mmol), allyltrimethylsilane (0.777 g, 1.08 mL) and TMSBr (2.603 g, 2.24 mL) in dry 1,2-dichloroethane (5 mL) was left for 3 h at RT under argon. Then, volatile components were removed under reduced pressure (0.5 mm/RT). The residue was dissolved in dry MeOH (20 mL) and the pH was brought to 7–8 by addition of aqueous 1 M NaOH. After cooling to −78 °C, ozone-containing dry air was bubbled through the stirred solution until a faint blue color persisted. The cooling bath was removed to allow the reaction mixture to warm up to RT. Ph_3P (30 mg, dissolved in 1 mL of CH_2Cl_2) was added, followed by water (40 mL) 5 min later. The mixture was extracted with CH_2Cl_2 (3 × 10 mL). The aqueous layer was neutralised (pH 7, 1 M NaOH) and lyophilised to yield

the solid sodium salt of (±)-**26**; ν_{max}/nujol mull/cm^{-1} 3300–2500, 1703, 1170, 1077; δ_H (400.1 MHz; D_2O) 4.63 (1 H, d, *J* 19.7, PCH), 2.31 (3 H, s, CH_3), contained 14 mol% of HCO_2Na (s at 8.42); δ_C (100.6 MHz; D_2O) δ 211.0, 79.0 (d, *J* 135.0), 27.7, formate: 120.8; δ_P (162 MHz; D_2O) 10.8, P_i at 0.9 (ratio 100 : 7).

Preparation of diethyl trimethylsilyl [$^{18}O_1$]phosphite. A solution of freshly distilled triethyl phosphite (8.30 g, 50 mmol), dry THF (5 ml), and $H_2{}^{18}O$ (1.0 g, 90% ^{18}O) was kept for 16 h at room temperature, then concentrated under reduced pressure and bulb to bulb distilled (70 °C/8 mm) to give diethyl [P(^{18}O)]phosphite[33] (6.34 g, 91%); $n_D{}^{20}$ 1.4071 (unlabelled compound: $n_D{}^{20}$ 1.4065). A mixture of the labelled phosphite (6.34 g, 45.3 mmol), hexamethyldisilazane (3.66 g, 4.79 mL, 22.7 mmol) and TMSCl (2.47 g, 2.90 mL, 22.7 mmol) and dry hexane (45 mL) was refluxed for 1 h. The precipitated NH_4Cl was removed by filtration through Celite. The solution was concentrated under reduced pressure and the residue was bulb to bulb distilled (75–80 °C/18 mm, lit.[32] 76–77 °C/20 mm for unlabelled compound) to give oxygen-18 labelled silyl phosphite (8.36 g, 87%) as a colourless liquid.

(*R*)-(+)-Isobutyl 2-(triisopropylsilyloxy)propionate [(*R*)-36]. *i*Pr_3SiCl (4.71 g 24.0 mmol, 5.32 mL) was added dropwise to a stirred solution of (*R*)-(+)-isobutyl 2-hydroxypropionate (2.92 g, 20.0 mmol) and imidazole (3.00 g, 44.0 mmol) in dry DMF (15 mL) at 0 °C under argon. The reaction mixture was stirred for 78 h at ambient and was then poured into a mixture of Et_2O (50 mL) and water (50 mL). The organic phase was separated. The aqueous layer was extracted with Et_2O (3 × 25 mL). The combined organic layers were washed with 1 N HCl (50 mL) and a saturated $NaHCO_3$ solution, dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by flash chromatography (hexanes–acetone 4 : 1, R_f 0.20) to give silylated ester (*R*)-**36** (5.70 g, 74%) as a colourless oil (Found: C, 64.0, H, 11.0. $C_{23}H_{32}O_3Si$ requires C, 63.5, H, 11.3%); $[\alpha]_D{}^{20}$ +24.4 (*c* 1.80 in acetone); ν_{max}/NaCl, film/cm^{-1} 2944, 2868, 1759, 1734, 1460, 1305, 1277, 1147; δ_H (400.1 MHz; $CDCl_3$) 4.41 (1 H, q, *J* 6.9, CHO), 3.88 (AB part of an ABX system, $J_{AB} = J_{BX} = 6.9$, J_{AB} 10.5, CO_2CH_2), 1.93 (1 H, nonett, *J* 6.9, CH), 1.41 (d, *J* 6.9, CH_3), 1.15–0.98 (21 H, m, TIPS), 0.92 (6 H, d, *J* 6.9, $C(CH_3)_2$); δ_C (100.6 MHz; $CDCl_3$) 174.3, 70.8, 68.5, 27.7, 21.9, 19.0, 17.9 and 17.8 (6 C), 12.1 (3 C).

(1*R*,2*R*) and (1*S*,2*R*)-Diethyl 2-triisopropylsilyloxy-1-hydroxypropyl[P=^{18}O]phosphonate [(1*R*,2*R*)- and (1*S*,2*R*)-38]. DIBAH (7.8 mL, 7.8 mmol, 1 M solution in *n*-heptane) was added dropwise to a stirred solution of silylated ester (*R*)-**36** (2.69 g, 7. 0 mmol) in dry Et_2O (25 mL) at –78 °C under argon. The reaction mixture was stirred for 2 h at –78 °C. Water (1.5 mL) was added and the mixture was stirred for 0.5 h at 0 °C. The aluminium hydroxide was removed by filtration through Celite and washed with Et_2O (50 mL). The combined solutions were dried ($MgSO_4$) and concentrated under reduced pressure. The residue dissolved in dry toluene, concentrated under reduced pressure and dried (0.50 mm/1 h) to yield the crude aldehyde (*R*)-**37** (1.61 g, quantitatively) as an oil.[31]

Diethyl ^{18}*O*-trimethylsilyl phosphite (1.78 g, 8.40 mmol) dissolved in dry toluene (5 mL) was added dropwise to the stirred solution of the crude aldehyde (1.61 g, 7.00 mmol) in dry toluene (20 ml) at –78 °C under argon. The reaction mixture was stirred for 16 h, while the bath was allowed to warm slowly to +10 °C. Volatile components were removed (finally at 0.50 mm/1 h) to yield a mixture of the crude silylated phosphonates as an oil. It was dissolved in ethanol (40 mL) and stirred at RT for 1 h after the addition of 4 drops of conc. HCl [TLC: hexanes–acetone 3 : 1, R_f 0.45 for (1*R*,2*R*)-**38** and R_f 0.36 for (1*S*,2*R*)-**38**].

The solution was concentrated under reduced pressure. Water (10 mL) was added to the residue and the mixture was extracted with EtOAc (3 × 15 mL). The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo.* The residue was purified by flash chromatography (hexanes–acetone 10 : 1) to give phosphonates (1*R*,2*R*)-**38** (1.28 g, 50%) and (1*S*,2*R*)-**38** (0.15 g, 6%) as colourless oils.

(1*R*,2*R*)-38. $[\alpha]_D{}^{20}$ –2.1 (*c* 2.0 in acetone) {lit.[31a] $[\alpha]_D{}^{20}$ –7.3 (*c* 1.2 in $CHCl_3$}. The spectroscopic data agree with that of the literature, except for the ^{31}P NMR spectrum (162 MHz, $CDCl_3$), which showed a resonance at 23.37 (P=O, 18%) (lit.[31a] 23.1) with a shoulder at 23.42 (P=^{18}O, 82%; isotope induced satellite signal); ν_{max}/Si, film/cm^{-1} 3384, 3081, 2941, 1464, 1382, 1216, 1031; δ_H (400.1 MHz; $CDCl_3$) 4.27 (1 H, m, PCCH), 4.15 (4 H, sext, *J* 7.0, 2 × OCH_2), 3.57 (1 H, t, *J* 6.0, CHP), 2.50 (1 H, bs, OH), 1.30 (3 H, d, *J* 6.0, CH_3), 1.28 and 1.27 (each 3 H, t, *J* 7.0, $OCCH_3$), 1.10–0.99 (21 H, m, TIPS); δ_C (100.6 MHz; $CDCl_3$) 72.9 (d, *J* 162.9), 68.3 (d, *J* 5.4), 62.9 (d, *J* 6.9), 62.4 (d, *J* 6.9), 21.4 (d, *J* 6.1), 18.1 and 18.0 (2 s, each 3 C), 16.4 (d, *J* 5.4), 12.7 (3 C); δ_P (162 MHz; $CDCl_3$) 23.37 (P=O) and 23.42 (P=O^{18}); 84% ^{18}O [FD-MS: m/z: 369 (19%), 371 (100%)].

(1*S*,2*R*)-38. ν_{max}/Si, film/cm^{-1} 3420, 2940, 2865, 1463, 1382, 1221, 1092, 1078, 1026; δ_H (400.1 MHz; $CDCl_3$) 4.27 (1 H, m, PCCH), 4.13 (4 H, m, 2 × OCH_2), 3.97 (1 H, dd, *J* 10.5, 3.0, PCH), 2.45 (1 H, bs, OH), 1.29 (3 H, d, *J* 7.0, CH_3), 1.28 (6 H, t, *J* 6.5, 2 × $OCCH_3$), 1.00 (21 H, m, *i*Pr_3Si); δ_C (100.6 MHz; $CDCl_3$) 72.8 (d, *J* 161.4), 68.5 (d, *J* 8.4), 62.6 (d, *J* 6.9), 62.4 (d, *J* 6.9), 19.0 and 18.5 (6 C), 17.7 (d, *J* 2.3), 16.5 (d, *J* 5.4), 16.4 (d, *J* 5.4), 12.3 (3 C).

(1*R*,2*R*)-Diethyl 1,2-dihydroxypropyl-[P=^{18}O]phosphonate [(1*R*,2*R*)-39]. A mixture of (1*R*,2*R*)-2-(triisopropylsilyloxy)-propyl-[P=^{18}O]phosphonate (1.11 g, 3.01 mmol), glacial acetic acid (16 mL) and water (4 mL) was stirred for 16 h at 90 °C (TLC: EtOAc-MeOH 20 : 1, R_f 0.36). Volatile components were removed under reduced pressure and the residue was purified by flash chromatography (EtOAc-MeOH 20 : 1) to give 1,2-dihydroxyphosphonate (1*R*,2*R*)-**39** (0.39 g, 62%) as a colourless oil; $[\alpha]_D{}^{20}$ –12.7 (*c* 2.60 in acetone); δ_H (400.1 MHz; $CDCl_3$) 4.13 (5 H, m, 2 × OCH_2 and CHCP), 3.60 (1 H, dd, *J* 7.0, 3.0, PCH), 3.31 (2 H, bs, 2 × OH), 1.29 (3 H, t, *J* 7.0, CH_3), 1.28 (3 H, t, *J* 7.0, CH_3), 1.24 (3 H, dd, *J* 6.5, 1.5, CH_3); δ_C (100.6 MHz; $CDCl_3$) 71.6 (d, *J* 159.1), 66.4 (d, *J* 2.3), 63.4 (d, *J* 6.9), 62.6 (d, *J* 7.7), 19.1 (d, *J* 10.7), 16.5 and 16.4 (2 d, *J* 5.3); δ_P (162 MHz; $CDCl_3$) 24.66 (P=^{16}O) with shoulder at 24.70 (P=^{18}O); 81% ^{18}O [FD-MS: m/z:213 (23%), 215 (100%)].

(1*R*,2*R*)-(1,2)-Dihydroxypropyl-[^{18}O]phosphonic acid, ammonium salt {[P^{18}O](1*R*,2*R*)-6}. Under an argon atmosphere TMSBr (3.55 g, 23.30 mmol, 3.06 mL) was added to a stirred solution of (1*R*,2*R*)-1,2-dihydroxypropyl-[P=^{18}O]phosphonate (1*R*,2*R*)-**39** (0.39 g, 1.85 mmol) and allyltrimethylsilane (1.07 g, 9.40 mmol, 1.50 mL) in dry 1,2-dichloroethane (7.5 mL). The

reaction mixture was stirred at RT for 16 h. Volatile components were removed under reduced pressure (RT to 40 °C/0.5 mm) and the residue was dissolved in a mixture (10 mL) of ethanol–water 1 :1 and concentrated again. The residue was dissolved in water (10 mL) and conc. ammonia (1.5 mL) was added. Lyophilisation gave the phosphonic acid [$P^{18}O$](1*R*,2*R*)-**6** as ammonium salt (0.23 g). Its NMR spectra recorded in D_2O were identical to the ones of (1*R*,2*R*)-**6** as ammonium salt, derived from fosfomycin. The ^{31}P NMR spectrum did not show an isotope-induced satellite signal.

Microorganism and culture conditions. *Rhizobium huakuii* PMY1 was isolated as previously described.[10] Cells were grown in batch cultures at 30 °C on an orbital shaker at 100 rpm in minimal medium (pH 7.0) of the following composition: 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[10], 0.05 g; BME essential amino acids solution, 20 mL/L (Sigma), and 1 mL each of trace element solution[10] and vitamin solution[10] in 1 L. Either filter-sterilised (0.22 μm) fosfomycin (5 mM) or those organophosphonates under test as potential pathway intermediates were added as sole carbon and phosphorus source. Microbial growth was measured by the increase in absorbance at 650 nm using a ATI-Unicam PU 8625 UV/VIS spectrophotometer while phosphate release into the culture supernatant was monitored *via* the method[36] of Fiske and SubbaRow.

Enzyme asssays

(i) *In vitro* C–P bond cleavage activity. Cell-extracts of fosfomycin-grown cells were prepared by sonication as previously described.[10] C–P bond cleavage activity was assayed at 30 °C by measuring the amount of phosphate liberated from the supplied organophosphate substrate. Unless otherwise stated the assay mixture (1 mL) consisted of: 50 mM Tris-HCl buffer (pH 7.0) and 5 mM organophosphonate substrate. The reaction was initiated by the addition of approximately 5 mg/mL of cell-free extract from fosfomycin grown cells, incubated for 60 min and terminated by the addition of 200 μL of 50% (w/v) trichloroacetic acid. After centrifugation, the phosphate released was measured *via* the method[36] of Fiske and SubbaRow. Activity was expressed as the mean of three replicates, in units of nanomoles of phosphate liberated per minute per milligram of protein.

(ii) Phosphite oxidase. Phosphite oxidase activity was assayed *via* the method[37] of Malacinski in cell-extracts of fosfomycin grown cells of *R. huakuii* PMY1. The assay mixture contained the following (l mL); 450 μL of 50 mM Tris-HCl buffer (pH 7.5); 300 μL of 10 mM NAD^+ and 200 μL of either 50 mM phosphorous acid or sodium phosphite. The reaction was initiated by the addition of approximately 5 mg/mL of crude cell-extract and the mixture incubated for 60 min at 30 °C. The reaction was terminated by the addition of 200 μL of 50% (w/v) trichloroacetic acid and the phosphate produced measured *via* the method[36] of Fiske and SubbaRow.

For all enzyme assays, controls containing no crude cell-extract were prepared to evaluate the degree of background phosphate release.

Protein assay

Protein concentration was determined by the method[38] of Bradford using bovine serum albumin as standard.

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