Biosynthesis of Natural Products with a P–C Bond. Part 8:[1] On the Origin of the Oxirane Oxygen Atom of Fosfomycin in *Streptomyces fradiae*

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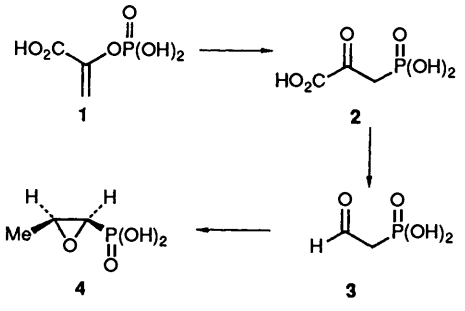
(*R,S*)-, (*R*)- and (*S*)-(2-Hydroxy-[1,1-$^2H_2$]propyl)phosphonic acid were fed to *Streptomyces fradiae* producing fosfomycin isolated as the corresponding ring-opened aminophosphonic acid. Only (*S*)-(2-hydroxy-[1,1-$^2H_2$]propyl)phosphonic acid was efficiently incorporated into fosfomycin (37% deuterium at C-1 of the aminophosphonic acid). (*R,S*)-(2-[^{18}O]Hydroxypropyl)phosphonic acid gave [1-^{18}O]aminophosphonic acid. None of the four stereoisomeric (1,2-dihydroxy-[1-$^2H_1$]-propyl)phosphonic acids labelled fosfomycin. Both (1*R*,2*S*)- and (1*S*,2*S*)-(1,2-dihydroxy-[1-^{3}H]-propyl)phosphonic acid were taken up into the cells, but were not specifically incorporated into fosfomycin, the dilution of radioactivity being 0.02 and 0.01%, respectively. Obviously they are not intermediates of the biosynthesis of fosfomycin.

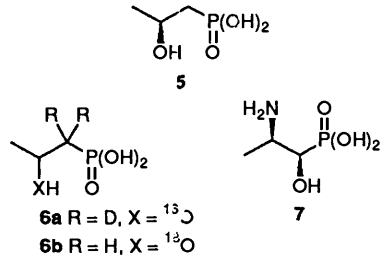
Fosfomycin[2] **4** is a clinically used antibiotic produced by various strains of *Streptomyces*, being a member of a small group of natural products[3] containing a P–C bond. It blocks the cell-wall biosynthesis of bacteria by alkylating an SH-group in the active site of phosphoenol-pyruvate: UDP-GlcNAc-enolpyruvyltransferase (suicide substrate analogue of phosphoenol pyruvate).[4] Fosfomycin is biosynthetically derived from a P–C_2-unit, most likely to be phosphonoacetaldehyde **3**, and a methyl group from L-methionine.[5] The P–C_2-unit is formed by intramolecular rearrangement[6] of phosphoenolpyruvate **1** to phosphonopyruvic acid **2** by the enzyme phosphoenolpyruvate mutase as shown[3c,7] for (2-aminoethyl)phosphonic acid and phosphinothricin, followed by decarboxylation (Scheme 1). Di[^{18}O]oxygen does not label the oxirane oxygen atom, thereby indicating that (*Z*)-(prop-1-enyl)phosphonic acid is not an intermediate.[8] (2-Aminoethyl)- and (2-hydroxyethyl)-phosphonic acid are incorporated into fosfomycin.[1,9,10]



Scheme 1

Results and Discussion

Studies with Deuterium- and Oxygen-18-labelled (2-Hydroxypropyl)phosphonic Acids.—In the previous paper[1] it was shown that one deuterium of (2-hydroxy-[1,1-$^2H_2$]ethyl)- as well as of (2-hydroxy-[2,2-$^2H_2$]ethyl)-phosphonic acid was retained on incorporation into fosfomycin *via* deuteriated phosphonoacetaldehyde. It was therefore suggested that the methyl group, for polarity reasons formally 'CH_3^-' (compare also ref. 11), is added to the carbonyl carbon of phosphonoacetaldehyde **3** to generate the P–C_3-unit of fosfomycin. In order to obtain more information on intermediates and the oxirane ring formation, labelled representatives[12] of the putative intermediate (2-hydroxypropyl)phosphonic acid **5** were fed to *Streptomyces fradiae*. The first experiment was carried out using racemic (2-hydroxy-[1,1-$^2H_2$]propyl)phosphonic acid **6a** as a precursor (Table 1, Exp. 1). It was obtained by passage of an aq. solution of the corresponding cyclohexylammonium salt[12] through Dowex 50 W (H^+-form). The free acid was added to the growth medium before autoclaving. The flasks were inoculated and shake cultured at 28 °C for *ca.* 60 h. Fosfomycin is a labile compound which is produced in low concentration, <10 mg dm^{-3} of broth, and cannot be isolated easily. It was therefore transformed by nucleophilic cleavage of the oxirane ring by ammonia mainly into (1*R*,2*R*)-(2-amino-1-hydroxypropyl)-phosphonic acid **7**, a stable derivative of fosfomycin. Work-up of culture broth for aminophosphonic acid **7** was as follows. The cells were centrifuged, and the supernatant was saturated with gaseous ammonia and kept for 3 days at 60 °C to form compound **7**, isolated by three-fold ion-exchange chromatography, following a published procedure.[8]



Fosfomycin production decreased on addition of precursor **6** to the medium as compared with a blank experiment without precursor **6** as shown in Table 1; the effect was more pronounced at even higher concentrations. The deuterium content at C-1 of aminophosphonic acid **7** was 44% as determined indirectly by ^{1}H NMR spectroscopy when the labelled precursor (*R,S*)-**6a** was fed to *Streptomyces fradiae*. The proton-noise-decoupled ^{13}C NMR spectrum (100.6 MHz) of the aminophosphonic acid **7**, recorded in D_2O, showed deuterium-induced[14,15] β- (0.067 ppm) and γ-shifted (0.032 ppm) satellite doublets for C-2 and C-3 at higher field, in agreement with values reported[1] for compound **7** with deuterium at C-1. The high incorporation rate demonstrated that one of the two enantiomers of racemic (2-hydroxy-[1,1-$^2H_2$]propyl)phosphonic acid **6a** was an excellent precursor and possibly a late intermediate in fosfomycin biosynthesis. Next, the enantiomeres[12] of compound **6a** were separately tested for incorporation into fosfomycin (Table 1, Expts. 2 and 3). Only isomer (*S*)-**6a**, having the same absolute configuration at C-2 as fosfomycin, was incorporated.

Table 1 Feeding experiments with labelled precursors **6**

Expt.	Substrate/quantity (μg cm^{-3})	Flasks[a]	Inhibition **5** (mm)[b]	Yield **7** (mg)[c]	% ^{2}H in **7**[d]
1	(*R,S*)-**6a**/100	6	16–18	2.5	44
2	(*R*)-**6a**/30	12	12–20	2.5	0
3	(*S*)-**6a**/30	12	21–25	5	37
4	(*R,S*)-**6b**/80	12	18–22	7	*e*

[a] Number of Erlenmeyer flasks (1000 cm^3) used as given in ref. 8, containing cornstarch medium (220 cm^3) with substrate to be tested for incorporation. [b] Diameter of inhibition zone for fosfomycin from individual flasks as determined[13] by agar-diffusion method with *Proteus mirabilis* NRRL B-3361 (disc size 9 mm diameter; diameter of blank 24 mm, corresponding to ~10 μg cm^{-3} of fosfomycin in broth). [c] Obtained according to ref. 8. [d] Determined indirectly by ^{1}H NMR (400 MHz) spectroscopy. [e] See Fig. 1.

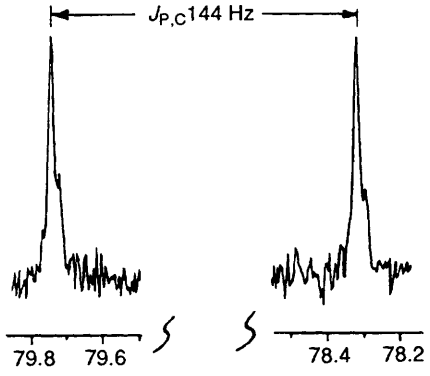
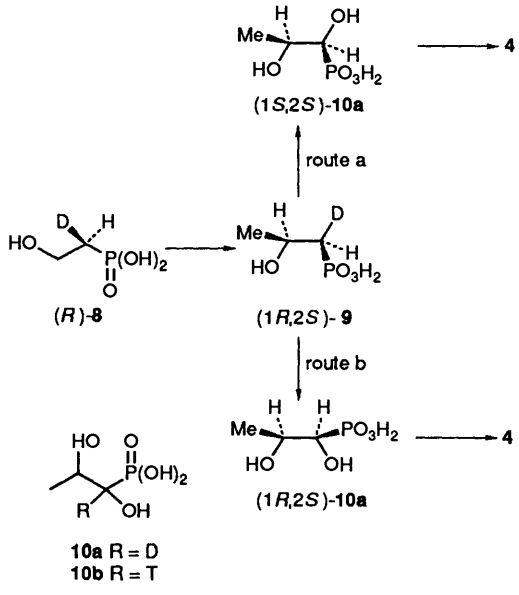


Fig. 1 Partial proton-noise-decoupled ^{13}C NMR spectrum (100.6 MHz) of aminophosphonic acid **7** labelled by (*R,S*)-(2-[^{18}O]hydroxypropyl)phosphonic acid **6b**; recorded in D_2O, containing NaOD, C-1 signal



Scheme 2

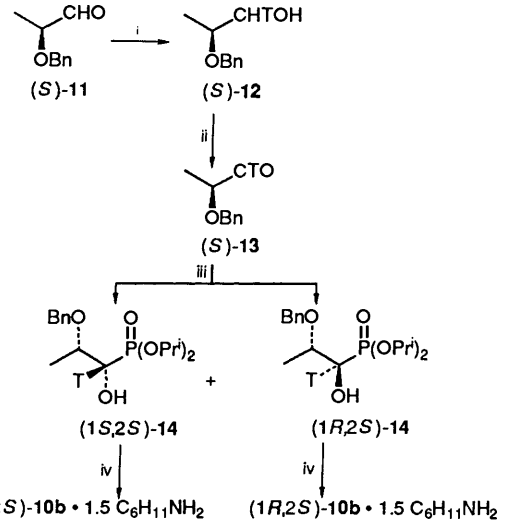
As the oxirane oxygen atom of fosfomycin was not derived from di[^{18}O]oxygen, it had to originate either from the water of the growth medium or from one of the oxygen atoms already present in (2-hydroxypropyl)phosphonic acid **5**. A plausible candidate was the oxygen atom of the hydroxy group at C-2 of compound **5**. (*R,S*)-(2-[^{18}O]Hydroxypropyl)phosphonic acid **6b**[12] (65% oxygen-18 enrichment at C-2) was used as a probe (Table 1, Expt. 4). The proton-noise-decoupled ^{13}C NMR spectrum (100.6 MHz) of aminophosphonic acid **7** isolated from the feeding experiment was recorded in D_2O before and after the addition of NaOD (30% in D_2O; under these conditions incorporation of deuterium does not take place). Only in the latter case did the doublet for C-1 [δ_C 79.03; J(P, ^{13}C) 144 Hz] show a satellite doublet shifted (oxygen-18-induced α-shift[15]) by 0.02 ppm to higher field (Fig. 1). The oxygen-18 content was estimated to be 20–25%. An authentic sample of compound **7** partially labelled with oxygen-18 at C-1 showed an α-shift of 0.025 ppm.[8] The origin of the oxirane oxygen atom was thus unequivocally confirmed to arise from the hydroxy group of hydroxyphosphonic acid **5**, which seems to have no precedence in biosynthetic epoxide formation. From the (*S*)-configuration at C-2 of compound **6a** it follows that the methyl group is added to the *pro-S* face of the carbonyl function of phosphonoacetaldehyde **3** to give propylphosphonic acid **5** with the (*S*)-configuration.

Studies with Deuterium- and Tritium-labelled (1,2-Dihydroxypropyl)phosphonic Acids.—From a chemical and biochemical point of view the biosynthetic transformation of (2-hydroxypropyl)phosphonic acid **5** into fosfomycin could entail hydroxylation at C-1 of the acid **5**, activation of the hydroxy group by, *e.g.*, phosphorylation, and displacement by oxygen of the OH group at C-2 to give the oxirane ring with inversion of configuration at C-1. It was demonstrated[1] by using chirally deuteriated (2-hydroxy-[1-^{2}H$_1$]ethyl)phosphonic acids that the deuterium of the (*R*)-enantiomer (*R*)-**8** was replaced by the C–O bond with net inversion of configuration at C-1, and the deuterium of the (*S*)-enantiomer was retained [Scheme 2, drawn only for (*R*)-**8**]. It was assumed that the (2-hydroxy-[1-^{2}H$_1$]ethyl)phosphonic acid was transformed by an oxidoreductase into the corresponding phosphonoacetaldehyde, the true intermediate. Taking this into account the most plausible intermediate could be (1,2-dihydroxypropyl)phosphonic acid (1*S*,2*S*)-**10a** formed by replacing the deuterium of (*R*)-**8** by a hydroxy group with retention of configuration (Scheme 2, route a). The alternative, replacing deuterium by hydroxy with inversion of configuration because of the neighbouring phosphoryl group, which would afford the (1,2-dihydroxypropyl)phosphonic acid (1*R*,2*S*)-**10a**, is very unlikely (Scheme 2, route b). The ensuing displacement of the activated OH group at C-1 would have to proceed with net retention of configuration. Such a mechanism could be operating despite a chemically unlikely,[16] but possibly enzymically feasible, participation of P=O as a neighbouring group to displace the activated OH group, which in turn is displaced by OH at C-2 with overall retention of configuration.

The synthesis and determination of absolute configuration of all four stereoisomeric (1,2-dihydroxy-[1-^{2}H$_1$]propyl)phosphonic acids **10a** with an enantiomeric excess of at least 98% from chiral lactates has been described in detail.[17] As these feeding experiments were already started at a time when the above mentioned findings were as yet unknown, all four stereoisomers were studied, and not just (1*S*,2*S*)-**10a** and (1*R*,2*S*)-**10a**. Three of them, (1*R*,2*R*)-, (1*R*,2*S*)- and (1*S*,2*R*)-**10a** at a concentration of 300 μg cm^{-3} of growth medium for *Streptomyces fradiae*, did not affect the amount of fosfomycin produced as compared with a blank experiment. The

aminophosphonic acids **7** isolated from the three experiments did not contain deuterium at a quantity detectable by ^1H NMR and, in part, ^{13}C NMR spectroscopy. The fourth isomer, (1*S*,2*S*)-**10a**, caused a decrease in the fosfomycin titre. At a concentration of 300 μg cm^{-3} the biosynthesis of fosfomycin was totally blocked; at a concentration of only 125 μg cm^{-3}, fosfomycin was produced at a level sufficient to isolate some of the derivative **7**. If deuterium is present in compound **7** at all, it is below the detection limit. This result is taken as evidence that at least (1*S*,2*S*)-**10a** was taken up into the cells, where the inhibition of fosfomycin biosynthesis occurred. Interestingly, the hydroxy group at C-1 of (1*S*,2*S*)-**10a** occupies the same stereochemical position as the deuterium in (1*R*,2*S*)-**9**, which will be removed at a later stage.

As small quantities (<5%) of deuterium in compound **7** cannot be detected by ^1H and ^{13}C NMR spectroscopy, tritiated diastereoisomers (1*S*,2*S*)- and (1*R*,2*S*)-**10b** were synthesized to repeat the above experiments with these more sensitive probes (Scheme 3). (S)-O-Benzyllactaldehyde (*S*)-**11**[18] was reduced



Scheme 3 *Reagents and conditions:* i, aq. MeOH, −15 °C, $NaBH_3T$; $NaBH_4$; ii, Swern oxidation; iii, THF, $(Pr^iO)_2PH(O)$, DBU, −78 to +20 °C; iv, deprotection (Me_3SiBr, Pd/C, H_2) and addition of cyclohexylamine as given in ref. 17

with sodium boro[^{3}H]hydride to afford the tritiated [1-^{3}H]-propanediol (*S*)-**12**, which was oxidised by the method of Swern to give tritiated aldehyde (*S*)-**13**.[17,19] 1,8-Diazabicyclo-[5.4.0]undec-7-ene (DBU)-catalysed addition of diisopropyl hydrogen phosphite to the tritiated aldehyde (*S*)-**13** occurred without racemisation in tetrahydrofuran (THF) at from −78 to +20 °C and afforded two diasteroisomeric α-hydroxypropyl-phosphonates **14** in roughly equal amounts (TLC). These were separated by flash chromatography, deprotected, and transformed into the corresponding cyclohexylammonium salts for purification as described[17] for the deuteriated compounds.

Two experiments were set up to determine both the incorporation of (1,2-dihydroxy-[1-^{3}H]propyl)phosphonic acids (1*R*,2*S*)- and (1*S*,2*S*)-**10b** into fosfomycin and uptake into *Streptomyces fradiae*. The concentration outside the cells was 300 μg cm^{-3} for (1*R*,2*S*)-**10b** and 50 μg cm^{-3} for (1*S*,2*S*)-**10b**. Both compounds were taken up into the cells. The ratios of concentration outside to inside were 100:3 and 100:4 for (1*R*,2*S*)- and (1*S*,2*S*)-**10b**, respectively. This corresponds to a concentration inside of 9 and 2 μg cm^{-3}, respectively. The two tritiated (1,2-dihydroxypropyl)phosphonic acids were therefore present at a concentration expected to lead to at least a few percentages of incorporation into fosfomycin, if they were specifically used as intermediates. The aminophosphonic acids **7** isolated from the culture broths showed a dilution of radioactivity (100 × specific activity of **7** derived from fosfomycin/specific activity of precursor, expressed in percentages) of 0.02 and 0.01% for (1*R*,2*S*)- and (1*S*,2*S*)-**10b**, respectively. Assuming that the ratio of (1*S*,2*S*)-**10b** outside to inside the cells is the same for 50 and 300 μg cm^{-3}, then at 2 μg cm^{-3} (77 μ mol dm^{-3}) inside the cells the biosynthesis of fosfomycin is totally inhibited.

Hydroxylation at C-1 of (2-hydroxypropyl)phosphonic acid **5** to generate a (1,2-dihydroxypropyl)phosphonic acid is not on the pathway to oxirane ring formation. Introduction of a still unknown functionality instead of hydroxy with retention of configuration and which can be displaced by oxygen of hydroxy at C-2 remains an unlikely possibility. The last step (steps) for the transformation of (2-hydroxypropyl)phosphonic acid **5** into fosfomycin remains (remain) open. In summary it is an oxidative process, consisting in removal of two hydrogens or an equivalent thereof. Oxidative cyclisations leading to four- and five-membered rings as encountered in the biosynthesis of both penicillin[20] and clavaminic acid[21] occur with retention of configuration at the carbon atom, where the bond to the heteroatom is formed. Further work will concentrate on trying to isolate the enzyme (enzymes) catalysing the last steps in fosfomycin biosynthesis.

Experimental

For general points, feeding experiments with *Streptomyces fradiae*, and isolation of the aminophosphonic acid **7** see ref. 8; ^1H and ^{13}C NMR spectra were recorded in D_2O on a Bruker AM 400 WB instrument. Chemical shifts for ^{13}C NMR are relative to sodium 4,4-dimethyl-4-silapentane-1-sulphonate (DSS) (δ 0.0). Radioactivity measurements were made on a Beckmann LS 1801 counter using Ecolume (ICN Biomedicals) as counting liquid.

(S)-2-*Benzyloxy*-[1-^{3}H]*propan*-1-*ol* (S)-**12**.—Crude aldehyde (*S*)-**11**, prepared[18] from (*S*)-ethyl *O*-benzyllactate (10 mmol) by reduction with diisobutylaluminium hydride (DIBAH), was dissolved in a mixture of methanol–water (25 cm^3–4 cm^3) and the solution was cooled to −15 °C (bath temperature). Sodium boro[^{3}H]hydride (4 mg, 50 mCi) was added and the solution was stirred for 15 min. Solid $NaHCO_3$ (0.2 g) and $NaBH_4$ (0.2 g) were added, and the mixture was stirred for 15 min at −15 °C and then until completion of reaction (20 min, TLC) at room temperature. Water (10 cm^3) was added, followed by 2 mol dm^{-3} HCl to acidify the solution. When evolution of hydrogen stopped, the product was extracted with CH_2Cl_2. The extract was dried (Na_2SO_4) and concentrated under reduced pressure. Bulb-to-bulb distillation afforded compound (*S*)-**12** (1.59 g, 96%) as an oil; b.p. 90 °C at 0.005 mmHg (lit.,[22] b.p. 101–101.5 °C at 1.2 mmHg for unlabelled racemic compound); 616 × 10^6 cpm mmol^{-1}.

(1S,2S)- *and* (1R,2S)-*Diisopropyl* (2-*Benzyloxy*-1-*hydroxy*-[1-^{3}H]*propyl*)*phosphonate* (1S,2S)- *and* (1R,2S)-**14**.—These compounds were prepared by analogy to the deuteriated compounds.[17] The tritiated alcohol (*S*)-**12** (1 g, 6 mmol) was transformed by Swern oxidation into the aldehyde (*S*)-**13**. A solution of crude aldehyde (*S*)-**13** in dry THF (7 cm^3) was added dropwise to a stirred solution of diisopropyl hydrogen phosphite (1.5 cm^3) and DBU (3 drops) in dry THF (8 cm^3) at −78 °C with exclusion of moisture. The mixture was stirred and allowed to warm up gradually to 20 °C. Acetic acid (3 drops) was added. Solvent and unchanged diisopropyl hydrogen phosphite were removed under reduced pressure (rotary evaporator, then at 0.05 mmHg up to 45 °C). The diastereoisomers were separated by flash chromatography; combined yield of (1*S*,2*S*)- and (1*R*,2*S*)-**14** (1.385 g, 70%); pure

(1*S*,2*S*)-**14** (0.525 g) had m.p. 87 °C (from hexane) (lit.,[17] 85 °C); $[\alpha]_D^{20}$ +22.9° (*c* 1.45 in CH_2Cl_2) {lit.,[17] $[\alpha]_D^{20}$ +22.4° (*c* 1.6 in CH_2Cl_2)}; 508 × 10^6 cpm $mmol^{-1}$; pure (1*R*,2*S*)-**14** (0.68 g) had $[\alpha]_D^{20}$ +6.4° (*c* 1.35 in CH_2Cl_2) {lit.,[17] $[\alpha]_D^{20}$ +6.1 (*c* 2.03 in CH_2Cl_2)}; (507 × 10^6 cpm $mmol^{-1}$; mixture (0.18 g).

Preparation of Cyclohexylammonium Salts of the Phosphonic Acids (1S,2S)-*and* (1R,2S)-**10b**.—These compounds were prepared by the same procedure as for the deuteriated compounds.[17] (1*S*,2*S*)-**10b**·1.5 $C_6H_{11}NH_2$ (54%) had m.p. 165–70 °C (from EtOH–Et_2O) (lit.,[17] 162–165 °C). (1*R*,2*S*)-**10b**·1.5 $C_6H_{11}NH_2$ (80%) had m.p. 187–190 °C (from EtOH–Et_2O) (lit.,[17] 183–187 °C); 489 × 10^6 cpm $mmol^{-1}$.

Incorporation Studies and Determination of Ratios of Concentration Inside to Outside of Cells for (1R,2S)- *and* (1S,2S)-(1,2-*Dihydroxy*-[1-^{3}H]*propyl*)*phosphonic Acid*.—Four Erlenmeyer flasks (250 cm^3) containing growth medium (40 cm^3) with (1*R*,2*S*)-**10b** (300 μg cm^{-3}, 0.975 × 10^6 cpm cm^{-3}) were inoculated and shake cultured.[8] After 28 h, when the production of fosfomycin (10 μg cm^{-1}) had practically ceased, the cells from culture broths (4 × 30 cm^3) were centrifuged (5 min; 8000 rpm; 4 °C; *ca.* 4 g pellet from each) separately and washed (4 × 26 cm^3 for each pellet) with 0.8% sterile saline. The pooled supernatants from the first centrifugation were worked up for compound **7** (yield 30 mg) after the addition of nonradioactive compound **7** (30 mg) as carrier. A yield of 0.5 mg of the acid **7** should be derived from the fosfomycin produced, assuming a 50% yield for transformation of epoxide **4** into compound **7**.[8] Dilution of radioactivity after crystallisation of compound **7** was 0.02%. Parts of two pellets were separately hydrolysed for 18 h by refluxing with 6 mol dm^{-3} HCl. The solution was brought to dryness, dissolved in water, decolourised with charcoal, concentrated and taken up in water for counting. Blank experiments with cyclohexylammonium salts of (1*R*,2*S*)- and (1*S*,2*S*)-**10b** treated in the same way revealed that part of the radioactivity (76 and 27%, respectively) was lost due to decomposition. Taking this into account, the radioactivity of harvested cells was 30 × 10^3 cpm g^{-1} (direct counting of cells gave 21 × 10^3 cpm g^{-1}). Therefore the ratio of concentration of (1*R*,2*S*)-**10b** outside the cells to the concentration inside the cells is 100:3 (assuming that 1 g of wet cell mass has a volume of 1 cm^3), corresponding to 9 μg cm^{-3} inside *Streptomyces fradiae* at a concentration of 300 μg cm^{-3} outside.

A similar experiment was performed for (1*S*,2*S*)-**10b** (two 1 dm^{-3} Erlenmeyer flasks, each containing 220 cm^3 of medium with 50 μg cm^{-3} substrate; 0.16 × 10^6 cpm cm^{-3}). Here, before the onset of fosfomycin production, 27 h after inoculation, two 15 cm^3 portions were withdrawn, centrifuged, washed and treated as before. The ratio of concentrations of (1*S*,2*S*)-**10b** outside to inside the cells was 100:4, corresponding to 2 μg cm^{-3} in the cells at 50 μg cm^{-3} outside. The supernatant (2.5 μg cm^{-3} of compound **4**) from one flask, obtained by centrifugation after 60 h, was worked up for compound **7** as before. The dilution of radioactivity was 0.01%.

Acknowledgements

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