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On the conversion of structural analogues of (*S*)-2-hydroxypropylphosphonic acid to epoxides by the final enzyme of fosfomycin biosynthesis in *S. fradiae*

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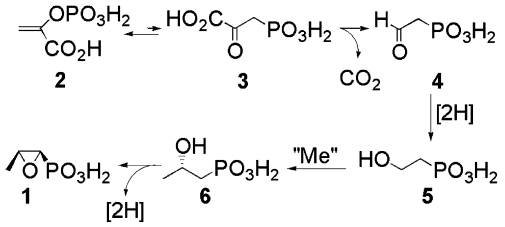
Abstract—2-Hydroxyethyl- and (*S*)-2-hydroxybutylphosphonic acid were prepared, starting in the latter case from (*S*)-2-aminobutyric acid. They were fed to cultures of *Streptomyces fradiae* producing fosfomycin. Only the latter (150 μg/mL of medium) was converted to the ethyl analogue of fosfomycin, isolated as 2-amino-1-hydroxybutylphosphonic acid (3%) in admixture with 2-amino-1-hydroxypropylphosphonic acid (97%) derived from fosfomycin.

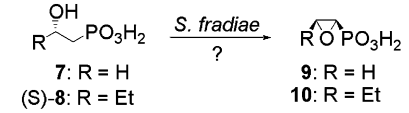
Fosfomycin [**1**, (1*R*,2*S*)-epoxypropylphosphonic acid] is a clinically utilized antibiotic of low toxicity, blocking bacterial cell wall biosynthesis by acting as an analogue of phosphoenolpyruvate (PEP).[1–3] It is one of the rare natural products containing a P–C bond,[4] produced by various species of *Streptomyces*,[5] *Pseudomonas syringae*, and *P. viridiflava*.[6,7] The biosynthesis of fosfomycin was unraveled by using feeding experiments with labeled precursors[8–12] and genetic[13] techniques. It comprises five steps, three of which are unique, starting from the primary metabolite PEP (**2**), which is rearranged reversibly by PEP mutase to give phosphonopyruvate (**3**) (Scheme 1).[14–16]

Decarboxylation[17] and reduction[18] produce 2-hydroxyethylphosphonic acid (**5**). The recently elucidated methylation of **5** producing (*S*)-2-hydroxypropylphosphonic acid (**6**, Hpp) follows a unique radical mechanism with SAM as methyl donor.[18,19] The final step is a dehydrogenative cyclization performed by a non-heme iron oxygenase [(*S*)-2-hydroxypropylphosphonic acid epoxidase, HppE].[20–24] Liu et al. found that the epoxidase converted (*R*)-2-hydroxypropylphosphonic and (*S*)-1,1-difluoro-2-hydroxypropylphosphonic acid to 2-oxopropylphosphonic acids, which has some bearing on the mechanism of the epoxide ring closure.[20]



Scheme 1. Biosynthesis of fosfomycin.

Inspired by the broad substrate specificity of isopenicillin *N* synthase,[25] also a non-heme iron dependent oxygenase, we decided to prepare analogues of Hpp, in the first place those with a hydrogen atom or an ethyl group replacing the methyl group in **6** to study the substrate specificity of the epoxidase. These homologues, **7** and **8**, were probed for their conversion to the analogous epoxides of fosfomycin by cultures of *Streptomyces fradiae* (Scheme 2). When this work was started, the epoxidase had not yet been purified and characterized. The cyclohexylammonium salt of 2-hydroxyethylphosphonic acid (**7**) was prepared by a literature procedure.[11] The



Scheme 2. Conversion of homologues of **6** to epoxides by *S. fradiae*.

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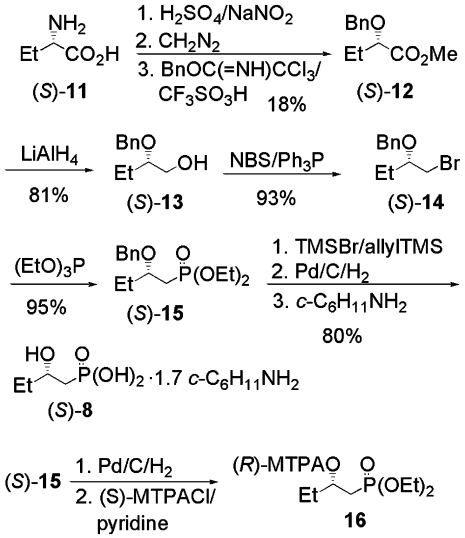
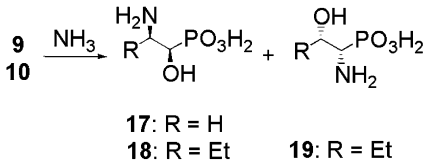


Scheme 3. Conversion of 2-aminobutyric acid to 2-hydroxyphosphonic acid (*S*)-**8** (as cyclohexylammonium salt).

synthesis of **8** is given in Scheme 3, starting from (*S*)-2-aminobutyric acid.[26,27] [(*S*)-**11**] as chiral precursor. The carboxyl group of the intermediate 2-hydroxyacid was esterified[26] and the hydroxyl group was benzylated[28] using Bundle's reagent to give ester (*S*)-**12** in an overall yield of 18%. Reduction of the ester (81%), conversion of the alcohol to the bromide (93%) followed by an Arbusov reaction with triethyl phosphite (95%) furnished 2-benzyloxyphosphonate (*S*)-**15**.[11,29] Removal of the protecting groups (TMSBr for Et on phosphorus, Pd/C/H_2 for Bn) gave phosphonic acid (*S*)-**8**, which was converted to the crystalline cyclohexylammonium salt for purification, containing 1.7 mol of amine as found by ^{1}H NMR spectroscopy. To establish the ee of the final product, a sample of phosphonate (*S*)-**15** was hydro-genolytically deprotected and esterified with (*S*)-MTPACl to yield Mosher ester **16**. Similarly, a reference sample of racemic **15**[30] was esterified. On the basis of the ^{1}H NMR spectra, the ee of (*S*)-**15** was found to be 98%. The salt of acid (*S*)-**8** should have the same ee, as the stereochemistry is not affected on deprotection of **15**.

Isolation of fosfomycin from the broth of *S. fradiae* was not possible because of its low concentration (up to 10 μg/mL) and its similar behavior to phosphoric acid (1 mg/mL of K_2HPO_4 in the medium) on ion exchange chromatography.[8] Therefore, the antibiotic was converted to two isomeric aminophosphonic acids by ring opening with ammonia, of which the (1*R*,2*R*)-2-amino-1-hydroxypropylphosphonic acid was amenable to isolation by ion exchange chromatography.[9] Assuming that the analogues **9** and **10** of fosfomycin are formed in smaller amounts than fosfomycin, if at all and then in admixture with it, we thought that treatment of the broth would yield amino-hydroxyphosphonic acids. The 2-amino-1-hydroxyphosphonic acids derived from fosfomycin and an analogue would behave similarly upon purification. NMR spectroscopy would allow the

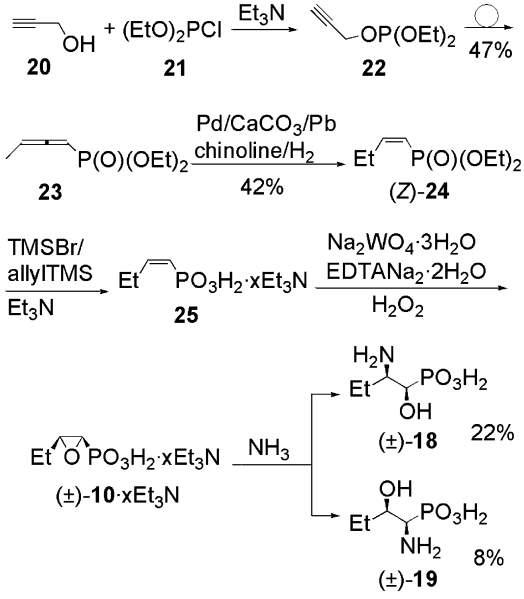


Scheme 4. Conversion of epoxides **9** and **10** to amino-hydroxyphosphonic acids by ammonia.

detection of the aminophosphonic acid **17** or **18** in the isolated mixture.

These arguments induced us to study the behavior of epoxides **9** and **10** toward ammonia and prepare reference compounds **17**–**19** (Scheme 4). Epoxide **9** will be opened by ammonia exclusively by attack at the less hindered site at C-2.[31] As 2-amino-1-hydroxyethylphosphonic acid[32] (**17**) was available from a previous project and its behavior was therefore known, it was not necessary to study the conversion of **9** to **17**.

However, the preparation of epoxide **10**, for convenience in the racemic form, and its conversion to amino-hydroxybutylphosphonic acids **18** and **19** had to be performed (Scheme 5). The used reactions are related to the ones used for the synthesis of fosfomycin, although some modifications had to be introduced.[33] Propargyl alcohol (**20**) was reacted with diethyl phosphorochloridite to phosphite **22** as intermediate, which underwent a smooth 2,3-sigmatropic rearrangement at ambient temperature to allenylphosphonate **23** in 47% yield.[34] The selective hydrogenation of one double bond was critical.[35] Using Pd/$CaCO_3$/Pb/H_2 (1 atm)/chinoline in dry ethanol furnished under optimized conditions at room temperature the desired (*Z*)-1-butenylphosphonate **23** (42%) and a mixture of (*E*)-**23** and possibly 2-butenylphosphonates (ratio 2:1, 20%) by flash chroma-



Scheme 5. Preparation of epoxide (±)-**10** and its ring opening with NH_3.

tography (hexanes/EtOAc 1:2). Removal of ethyl protecting groups at phosphorus effected by our standard method (TMSBr/allyltrimethylsilane) gave the free phosphonic acid, which was directly converted to the triethylammonium salt and epoxidized at 40 °C with H_2O_2 using $Na_2WO_4{\cdot}3H_2O$ as catalyst.[33] Anticipating difficulties with its purification, the racemic epoxide was not isolated, but heated with ammonia at 70 °C for 65 h. The two aminophosphonic acids (±)-**18** and (±)-**19** were isolated by ion exchange chromatography and obtained in a ratio of 2.5:1, identical with the ratio of the analogous aminophosphonic acids obtained from fosfomycin.[9] 2-Amino-1-hydroxypropylphosphonic acids derived from fosfomycin and **17** or (±)-**18** behaved similarly upon ion exchange chromatography, thus insuring co-isolation from the broth.

The first feeding experiment was performed with 2-hydroxyethylphosphonic acid (**7**) added to the growth medium of *S. fradiae* (six 1 L flasks with 220 mL of medium each, 500 μg/mL of 2-hydroxyethylphosphonic acid).[9] After 3 days, when the fosfomycin concentration was 15 μg/mL of broth, the cells were harvested and the supernatants were pooled, saturated with ammonia, and heated at 60 °C for 3 days. The 2-amino-1-hydroxypropylphosphonic acid was derived from fosfomycin possibly containing 2-amino-1-hydroxyethylphosphonic acid (**17**) was isolated (6 mg) by triple ion exchange chromatography. The 1-amino-2-hydroxypropylphosphonic was not retained by the first column of Dowex 50W,H^+. Surprisingly, **17** could not be detected in the product by NMR spectroscopy (^1H, ^{31}P), however 2% of (1*S*,2*R*)-2-amino-1-hydroxypropylphosphonic acid derived from *trans*-fosfomycin.[36] Previous feeding experiments with 1,1-dideuterated 2-hydroxyethylphosphonic acid (150 μg/mL) yielded 2-amino-1-hydroxypropylphosphonic acid with 32% D. As the samples were purified at that time by a final crystallization, the now looked for 2-amino-1-hydroxyethylphosphonic acid could not be found, even if it was present in the crude product.

Similarly, a second feeding experiment was set up with (*S*)-2-hydroxybutylphosphonic acid (**8**, 150 μg/mL) as substrate, obtained by passing down the cyclohexylammonium salt a column of Dowex 50W,H^+. The isolated mixture of aminophosphonic acids was very carefully investigated by ^1H, ^{31}P, and ^{13}C NMR spectroscopy. The signals of **18** were clearly detectable in all three spectra. The ratio of **18**, 2-amino-1-hydroxypropylphosphonic acid derived from fosfomycin, and the 2-aminophosphonic acid derived from *trans*-fosfomycin as determined by ^{31}P NMR spectroscopy was 3:95:2. When 1,1-dideuterated **6** (30 μg/mL) was fed to *S. fradiae*, the isolated 2-amino-1-hydroxypropylphosphonic acid contained 37% of the labeled species.[11]

The missing detection of 2-amino-1-hydroxyethylphosphonic acid (**17**) as proof for the formation of epoxide **9** and the very low incorporation of **8** into epoxide **10** do not necessarily indicate that the Hpp epoxidase is a highly substrate specific enzyme. The former compound, the substrate for the methylase, could probably be used too efficiently in the methylation reaction to be used by the epoxidase. Alternatively, epoxide **9** could be much more prone to chemical hydrolysis than fosfomycin or **10**, thus escaping transformation to aminophosphonic acid **17** with ammonia. One really would have to compare the reactions of these two substrates with the reaction of (*S*)-2-hydroxypropylphosphonic acid with the isolated epoxidase in vitro. The whole cell feedings address a range of factors, not just the substrate specificity of the epoxidase.

In summary, we have prepared the lower and higher homologue of Hpp and fed it to *S. fradiae*. Evidently, only the latter, the (*S*)-2-hydroxybutylphosphonic acid, was converted to the analogue of fosfomycin, being present in the broth as proven by conversion to aminophosphonic acids. The 2-aminophosphonic acids were isolated and identified by NMR spectroscopy and found to be present in a ratio of 3:95 derived from **10** and **1**, respectively.

Note added in proof: After submitting the manuscript, a paper by Liu et al.[37] was published, reporting on the determination of the substrate binding mode to the active site iron of (*S*)-2-hydroxypropylphosphonic acid epoxidase using[17] O-enriched substrates and substrate analogues [isobutyl-, (*R*)- and (*S*)-2-hydroxy-2-phenyl-, and (*R*)- and (*S*)-3-hydroxybutylphosphonic acid]. (*S*)-2-Hydroxy-2-phenylethylphosphonic acid was converted to the epoxide by the isolated oxygenase, albeit at much reduced rate. (*S*)-3-Hydroxybutylphosphonic acid furnished (*S*)-3,4-dihydroxybutylphosphonic acid and both (*R*)-enantiomers gave the corresponding keto-phosphonic acids.

Acknowledgment

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Supplementary data

All experimental procedures and spectroscopic data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.12.012.

References and notes

1. Kahan, F. M.; Kahan, J. S.; Cassidy, P. J.; Kropp, H. *Ann. N. Y. Acad. Sci.* **1974**, *235*, 364.
2. Skarzynski, T.; Mistry, A.; Wonacott, A.; Hutchinson, S. E.; Kelly, V. A.; Duncan, K. *Structure* **1996**, *4*, 1465.
3. Ribes, S.; Taberner, F.; Domenech, A.; Cabellos, C.; Tubau, F.; Linares, J.; Viladrich, P. F.; Gudiol, F. *J. Antimicrob. Chemother.* **2006**, *57*, 931.
4. Seto, H.; Kuzuyama, T. *Nat. Prod. Rep.* **1999**, *16*, 589.
5. Hendlin, D.; Stapley, E. O.; Jackson, M.; Wallick, H.; Miller, A. K.; Wolf, F. J.; Miller, T. W.; Chaiet, L.;

Kahan, F. M.; Foltz, E. L.; Woodruff, H. B.; Mata, J. M.; Hernandez, S.; Mochales, S. *Science* **1969**, *166*, 122.
6. Shoji, J.; Kato, T.; Hinoo, H.; Hattori, T.; Hirooka, K.; Matsumoto, K.; Tanimoto, T.; Kondo, E. *J. Antibiot.* **1986**, *39*, 1011.
7. Katayama, N.; Tsubotani, S.; Nozaki, Y.; Harada, S.; Ono, H. *J. Antibiot.* **1990**, *43*, 238.
8. Rogers, T. O.; Birnbaum, J. *Antimicrob. Agents Chemother.* **1974**, *5*, 121.
9. Hammerschmidt, F.; Bovermann, G.; Bayer, K. *Liebigs Ann. Chem.* **1990**, 1055.
10. Hammerschmidt, F. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 341.
11. Hammerschmidt, F.; Kählig, H. *J. Org. Chem.* **1991**, *56*, 2364.
12. Peric Simov, B.; Wuggenig, F.; Lämmerhofer, M.; Lindner, W.; Zarbl, E.; Hammerschmidt, F. *Eur. J. Org. Chem.* **2002**, 1139.
13. Hidaka, T.; Goda, M.; Kuzuyama, T.; Takei, N.; Hidaka, M.; Seto, H. *Mol. Gen. Genet.* **1995**, *249*, 274.
14. McQueney, M. S.; Lee, S.; Swartz, W. H.; Ammon, H. L.; Mariano, P. S.; Dunaway-Mariano, D. *J. Org. Chem.* **1991**, *56*, 7121.
15. Seidel, H. M.; Freeman, S.; Schwalbe, C. H.; Knowles, J. R. *J. Am. Chem. Soc.* **1990**, *112*, 8149.
16. Hidaka, T.; Iwakura, H.; Imai, S.; Seto, H. *J. Antibiot.* **1992**, *45*, 1008.
17. Nakashita, H.; Watanabe, K.; Hara, O.; Hidaka, T.; Seto, H. *J. Antibiot.* **1997**, *50*, 212.
18. Kuzuyama, T.; Hidaka, T.; Kamigiri, K.; Imai, S.; Seto, H. *J. Antibiot.* **1992**, *45*, 1812.
19. Woodyer, R. D.; Li, G.; Zhao, H.; van der Donk, W. A. *J. Chem. Soc., Chem. Commun.* **2007**, 359.
20. Zhao, Z.; Liu, P.; Murakami, K.; Kuzuyama, T.; Seto, H.; Liu, H. *Angew. Chem., Int. Ed.* **2002**, *41*, 4529.
21. Liu, P.; Mehn, M. P.; Yan, F.; Zhao, Z.; Que, L., Jr.; Liu, H. *J. Am. Chem. Soc.* **2004**, *126*, 10306.
22. McLuskey, K.; Cameron, S.; Hammerschmidt, F.; Hunter, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 14221.
23. Higgins, L. J.; Yan, F.; Liu, P.; Liu, H.; Drennan, C. L. *Nature* **2005**, *437*, 834.
24. Yan, F.; Munos, J. W.; Liu, P.; Liu, H. *Biochemistry* **2006**, *45*, 11473.
25. Baldwin, J. E.; Bradley, M. *Chem. Rev.* **1990**, *90*, 1079.
26. Mori, K.; Sasaki, M.; Tamada, S.; Suguro, T.; Masuda, S. *Tetrahedron* **1979**, *35*, 1601.
27. Chenault, H. K.; Kim, M.-J.; Akiyama, A.; Miyazawa, T.; Simon, E. S.; Whitesides, G. M. *J. Org. Chem.* **1987**, *52*, 2608.
28. Widmer, U. *Synthesis* **1987**, 568.
29. Hammerschmidt, F. *Monatsh. Chem.* **1991**, *122*, 389.
30. Mikołajczyk, M.; Bałczewski, P. *Synthesis* **1984**, 691.
31. Wróblewski, A. E.; Hałajewska-Wosik, A. *Tetrahedron: Asymmetry* **2000**, *11*, 2053.
32. Hammerschmidt, F.; Lindner, W.; Wuggenig, F.; Zarbl, E. *Tetrahedron: Asymmetry* **2000**, *11*, 2955.
33. Glamkowski, E. J.; Gal, G.; Purick, R.; Davidson, A. J.; Sletzinger, M. *J. Org. Chem.* **1970**, *35*, 3510.
34. Mark, V. *Tetrahedron Lett.* **1962**, *3*, 281.
35. Petrov, A. A.; Ionin, B. I.; Ignatyev, V. M. *Tetrahedron Lett.* **1968**, *9*, 15.
36. Woschek, A.; Wuggenig, F.; Peti, W.; Hammerschmidt, F. *ChemBioChem* **2002**, *3*, 829.
37. Yan, F.; Moon, S.-J.; Liu, P.; Zhao, Z.; Lipscomb, J. D.; Liu, A.; Liu, H. *Biochemistry* **2007**, *46*, 12628.