Digestion of Rhizocticins to (*Z*)-L-2-amino-5-phosphono-3-pentenoic acid: Revision of the Absolute Configuration of Plumbemycins A and B

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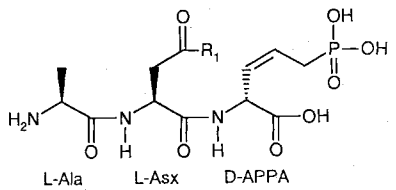
The rhizocticins[1] and plumbemycins[2] are two groups of di- and tripeptide antibiotics thought to interfere with threonine or threonine-related metabolism. Both contain the unusual amino acid (*Z*)-2-amino-5-phosphono-3-pentenoic acid (APPA). Rhizocticin A (**5**), B (**6**), C and D (**7**) were isolated as antifungal phosphono-oligopeptides from the culture broth of *Bacillus subtilis* ATCC 6633. They contain APPA (**8**) in the L-configuration which was shown by gas chromatography of a derivative on a chiral stationary phase[1]. At that time insufficient material was isolated for a determination of the $[\alpha]_D$-value. The plumbemycins A (**1**) and B (**2**), isolated as antagonists of L-threonine from *Streptomyces plumbeus*, were reported to have APPA in the D-configuration[3]. The absolute configuration was based on the CLOUGH-LUTZ-JIRGENSONS rule which indicated the D-configuration of amino acids[4]. In this paper an efficient digestion of rhizocticins to L-APPA, additional physico-chemical data of L-APPA (**8**) and the identity of the absolute stereochemistry of APPA from rhizocticin and plumbemycin are reported.

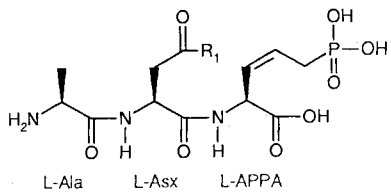
Digestion from the tripeptide rhizocticin B (**6**) to the dipeptide rhizocticin A (**5**) with pronase from *Streptomyces griseus* was completed in 15 minutes[1]. The enzymatic conversion to L-APPA needed high concentration of enzyme (10%) and several days. When testing for suitable conditions papain, a non-specific protease[5] from *Carica papaia*, was found to be as efficient as thermolysin[1] but much cheaper. Because of the long reaction time, the enzyme was stabilized by a high concentration of 2-mercaptoethanol. A volatile buffer was chosen for easier purification and pH 6 lead to higher yields than the commonly used NH_4HCO_3 buffer (pH 7.9). Chromatography on a cation exchanger and crystallization gave pure L-APPA (**8**).

The material gave identical ^1H NMR, IR and FAB-MS data as reported[1] and satisfactory elemental analysis. The optical purity of **8** was determined on Crownpak CR as 99.7% ee[6]. On this HPLC column, containing a chiral crown ether, the D-form of amino acids elutes before the L-form. The assignment of the absolute stereochemistry of RAPP *et al.*[1] was confirmed, as the peak of epimerized amino acid, obtained by heating in water, eluted before the one of natural APPA. The optical rotation of **8** was determined as +189° which is the same direction as the one of APPA from plumbemycins determined by PARK *et al.*[3] (+51.4°) but a much higher value. Consequently both, rhizocticins and plumbemycins, contain L-APPA as unusual amino acid and the absolute stereochemistry of plumbemycins A and B has to be revised to structures **3** and **4**. The higher $[\alpha]_D$-value obtained by enzymatic digestion suggests that partial racemization occurs when L-APPA is liberated by hydrolysis in 6N HCl. The pH dependency of the

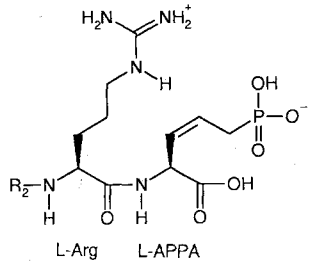
Fig. 1. Structures of plumbemycins A (**1**) and B (**2**) proposed by PARK *et al.*[2] and revised stereochemistry of plumbemycins A (**3**) and B (**4**). Structures of rhizocticins A (**5**), B (**6**) and D (**7**) and of L-APPA (**8**).



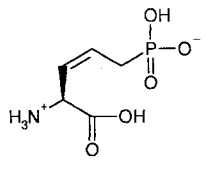
1 R_1 = OH
2 R_1 = NH_2



3 R_1 = OH
4 R_1 = NH_2



5 R_2 = H
6 R_2 = $(CH_3)_2CH\text{-}CH(NH_2)\text{-}CO$ (L-Val)
7 R_2 = $(CH_3)_2CH\text{-}CH_2\text{-}CH(NH_2)\text{-}CO$ (L-Leu)



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epimerization of L-APPA was investigated (Table 1). As expected by the adjacent double bond epimerization takes place much quicker than with simple aliphatic amino acids. The fast epimerization at pH 2.5 can be explained by a neighboring participation[7] of the spatially very close anionic phosphono group. In hydrochloric acid this group is protonated and the speed of the epimerization is drastically reduced.

The CLOUGH-LUTZ-JIRGENSONS rule predicts the absolute configuration of amino acids by the difference of their optical rotations in water and 5 N HCl as solvent[4]. If the rotation is shifted towards a more positive direction upon the addition of acid to its aqueous solution, the amino acid has the L-configuration. Contrary to PARK *et al.*[3] we find almost no difference between the $[\alpha]_D$-values in water and in 5 N HCl. A possible reason for the difference might be that our solution of APPA is quite acidic (pH 2.5). At pH 7 in phosphate buffer the $[\alpha]_D$-value is more positive. If the value at pH 7 is taken as a reference point, the CLOUGH-LUTZ-JIRGENSONS rule would indicate, just as the values from PARK *et al.*, the wrong absolute configuration.

NATCHEV synthesized L-APPA and plumbemycins A and B starting from (*E*)-4-bromocrotonaldehyde[8]. The drawings in his paper are ambiguous concerning the conformation of the double bond and the Bulgarian author never claims to have compounds in the cis conformation. From his synthetic route clearly he described (*E*)-2-amino-5-phosphono-3-pentenoic acid in his work having the double bond in trans conformation. Therefore the optical rotation values for APPA found by him are not relevant for our work.

The revised structure of the plumbemycins shows that they are closely related to the rhizocticins and support the mechanism that both act as L-threonine antagonist within the cell[9] possibly by inhibiting the threonine synthase[10]. The amino acids bound on the N-terminal of L-APPA are necessary for the efficient uptake of the antibiotics into the target cell and decide that the plumbemycins have antibacterial and the rhizocticins antifungal activity.

Experimental

Digestion of Rhizocticins and Purification of L-APPA

A mixture of 3 g of rhizocticins containing 4.6% rhizocticin A (**5**), 37% rhizocticin B (**6**) and 11% rhizocticin D (**7**) per weight (determined by amino acid analysis of the hydrolyzate) were dissolved in 300 ml pyridine-acetate buffer pH 6.0 (50 mM, 0.84 ml AcOH in H_2O and approx 6 ml pyridine). 2-Mercaptoethanol (0.5 ml) and papain (Fluka, 3.2 U/mg, 160 mg) were added and the solution was kept at 37°C for 56 hours. After 47 hours an additional portion of 40 mg papain was added. The enzyme was adsorbed by stirring the solution with Amberlite XAD-7 (20 ml, 20~50 mesh) for 15 minutes. The resin was removed by filtration and the solution lyophilyzed giving 3.82 g of an almost colorless material. This material was dissolved in 6 ml 1 N AcOH and loaded on a cation exchange column filled with AG 50W-X8 (H^+-form, 100~200 mesh, 78 ml). The column was eluted with 1 N AcOH (330 ml) and then with 3 N AcOH (240 ml) and fractions from 133 ml to 570 ml were collected and lyophilized yielding 805 mg of a colorless material. Pure **8** was obtained by crystallization in H_2O (12 ml) giving 627 mg as colorless crystals (91% yield).

Data of **8**

Anal Calcd for $C_5H_{10}NO_5P$:
C 30.78, H 5.17, N 7.18, P 15.88.
Found:
C 30.85, H 5.20, N 7.21, P 15.90.

FAB-MS *m/z* 196 (M+H).

UV λ^{H_2O} (ε) end absorption at 200 nm: 5800.

IR (KBr) cm^{-1} 3110, 3040, 2930, 1740, 1640, 1590, 1520, 1440, 1360, 1300, 1260, 1240, 1215, 1155, 1070, 1050, 970, 920, 750, 710.

^1H NMR (400 MHz, D_2O) δ 6.11 (1H, m, J=13, 11, 9 Hz, H-4), 5.66 (1H, ≈td, J=11, 10, 6 Hz, H-3), 4.82 (1H, d, J=10 Hz, H-1), 2.8~2.6 (2H, m, $J_{5a\text{-}5b}$=15 Hz, J=22, 9 Hz, H-5).

Table 1. pH profile of the epimerization of L-APPA (**8**) at 100°C.

buffer	pH	% D-APPA	
		30 minutes	2 hours
6 N HCl		2	7
1 N HCl	0.1	2	9
without buffer	2.55	8	25
25 mM ammonium acetate	5.05	4	NT
25 mM phosphate	7.05	3.3	10
25 mM borate	8.99	4	17
0.05N NaOH	12.58	21	NT

NT: not tested.

CD-spectrum (MeOH): nm (θ) 199 (+101,000).
CD-spectrum (H_2O): nm (θ) 203 (+98,000).
$[\alpha]_D^{20}$ (*c* 0.76, H_2O) = +189° +/−1.3°.
$[\alpha]_D^{20}$ (*c* 0.77, 5 N HCl) +192°.
$[\alpha]_D^{20}$ (*c* 0.78, 25 mM phosphate buffer pH 7.05) +241°.

Determination of the Enantiomeric Purity of APPA

The enantiomeric purity of **8** was determined on Crownpak CR(+) (4 × 150 mm, Daicel, Tokyo, Japan) cooled to 5°C, using aq 0.01 N $HClO_4$ of pH 1.96 as mobile phase with a flow rate of 0.4 ml/minute and UV detection at 200 nm. The sample was dissolved, at a concentration of 1 mg/ml, in water and 10 ml were injected (Rt D-APPA: 3.2 minutes; Rt L-APPA 5.2 minutes; Rt D-2-amino-5-phosphonovaleric acid (DL-mixture from Sigma): 3.4 minutes; Rt L-2-amino-5-phosphonovaleric acid: 4.7 minutes).

pH Dependency of the Epimerization of L-APPA (**8**)

A solution of **8** in the buffers found in Table 1, at a concentration of 1 mg/ml, was heated for 30 minutes or for 2 hours at 100°C. The 0.05 N NaOH solution was neutralized with 0.1 N HCl. The aq HCl was removed *in vacuo* and the sample dissolved in the same volume of H_2O. Of these solutions, 10 μl were injected into the HPLC with the following conditions: Crownpak CR(+), 4 × 150 mm, cooled to 0°C, using aq 0.1 N $HClO_4$ of pH 1.00 as mobile phase with a flow rate of 0.4 ml/minute and the detection wavelength set at 205 nm.

Acknowledgments

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