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R,S-Adenallene 4'-Phosphate: Substrate Activity and Enantioselectivity Toward AMP Deaminase and 5'-Nucleotidase

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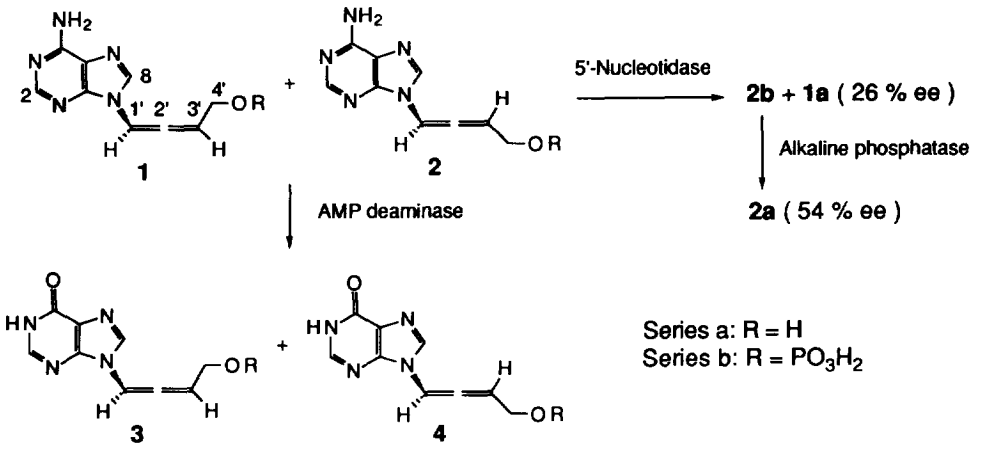
Abstract. R,S-Adenallene 4'-phosphate **1b** + **2b**, a putative metabolite of anti-HIV agent adenallene, is deaminated with AMP deaminase to R,S-hypoxallene 4'-phosphate **3b** + **4b** without detectable enantioselectivity. The dephosphorylation catalyzed by 5'-nucleotidase is slow but enantioselective giving R-adenallene **1a** (26% ee) and S-adenallene 4'-phosphate **2b** (54% ee).

R,S-Adenallene **1a** + **2a**, an unusual analogue of 2',3'-dideoxyadenosine with axial dissymmetry, is a strong inhibitor of the replication and cytopathic effect[1] of HIV-1 and -2. The antiretroviral activity of **1a** + **2a** is enantioselective[2] and the R-enantiomer **1a** is the most potent species. It is assumed that the mechanism of anti-HIV effect of R-adenallene **1a** is similar to that of 2',3'-dideoxyadenosine.[3] Thus, R-adenallene **1a** undergoes an intracellular phosphorylation, ultimately to the respective triphosphate, which then functions as a terminator of DNA chain in a reaction catalyzed by reverse transcriptase. It is therefore of interest to examine phosphorylated derivatives of R,S-adenallene **1a** + **2a** and their behavior toward enzymes of nucleic acid metabolism. The synthesis of the first phosphate of an allene comprising a nucleic acid base, R,S-adenallene 4'-phosphate **1b** + **2b**, its substrate activity and enantioselectivity toward AMP deaminase and 5'-nucleotidase is the subject of this communication.

Compound **1b** + **2b** was obtained by phosphorylation of R,S-adenallene **1a** + **2a** with 2-cyanoethyl phosphate in pyridine[4] and subsequent deprotection with NH_4OH in 30% yield. The R,S-adenallene 4'-phosphate **1b** + **2b** was deaminated quantitatively by AMP deaminase from rabbit muscle at 25°C and pH 6.5 in 24 h to give R,S-hypoxallene 4'-phosphate **3b** + **4b** as shown by UV spectra and paper electrophoresis (Scheme 1). Shortening of the reaction time to 5 h led to ca. 50% conversion to **3b** + **4b**. Digestion with alkaline phosphatase afforded a mixture of allenes **1a** + **2a** and **3a** + **4a** which was chromatographed on a Chiracel CA-1 column.[2] The ratio of R,S-hypoxallene **3a** + **4a** (this enantiomeric mixture is not separable),[2] R-adenallene **1a** and S-adenallene **2a** was 2 : 1 : 1 indicating no enantioselective deamination (Figure 1). By contrast, deamination of R,S-adenallene **1a** + **2a** with adenosine deaminase from calf intestine proceeded, under

Scheme 1



similar conditions (50% conversion), with a high[1,2,5] enantioselectivity for the S-enantiomer **2a**. Nevertheless, longer reaction time led to a complete deamination[5] of both **1a** and **2a**. In murine leukemia L1210 cells where R,S-adenallene **1a** + **2a** is not phosphorylated a non-enantioselective deamination constitutes a major metabolic pathway.[6] Our results indicate that deamination of R,S-adenallene 4'-phosphate **1b** + **2b** catalyzed by AMP deaminase may form a part of metabolism of adenallene **1a** + **2a** in cells capable of phosphorylating the analogue.

The behavior of adenallene 4'-phosphate **1b** + **2b** toward 5'-nucleotidase from the *Crotalus atrox* venom was also investigated. This enzyme was recently used for a preparative resolution[7] of anti-HIV agent BCH189 /(±)-2',3'-dideoxy-3'-thiacytidine/. The kinetics of dephosphorylation of **1b** + **2b** at pH 9 and 37°C was followed by paper electrophoresis and HPLC for 116 h (Figure 2). Decomposition was noted after 100 h of incubation. A 50% conversion to adenallene was reached after 40 h. Adenallene **1a** + **2a** and adenallene 4'-phosphate **1b** + **2b** were separated by paper electrophoresis. Chiral HPLC of the obtained adenallene showed it was predominatly the R-enantiomer **1a** (26% enantiomeric enhancement, ee). Adenallene 4'-phosphate **1b** + **2b** was degraded to adenallene **1a** + **2a** by alkaline phosphatase. Chiral chromatography indicated that the S-enantiomer **2a** (54% ee) was predominant. Hence, the R-phosphate **1b** is preferentially hydrolyzed by 5'-nucleotidase. It is interesting to note that a reverse reaction (phosphorylation), important for the anti-HIV activity[1] of adenallene **1a** + **2a**, may also exhibit enantioselectivity for the most active[2] R-enantiomer **1a**.

We can conclude that although adenallene 4'-phosphate **1b** + **2b** is a substrate for both AMP deaminase and 5'-nucleotidase, its enantioselectivity toward both enzymes differs significantly. This is another example that

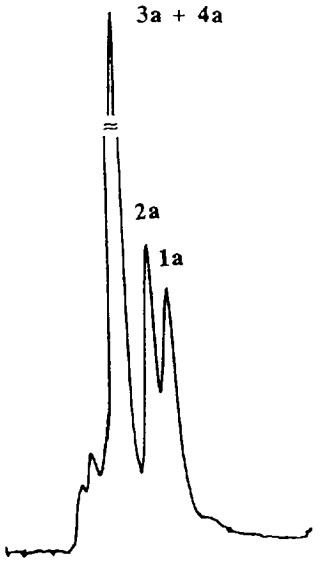


Figure 1. Chiral HPLC of the products of AMP-deaminase-catalyzed deamination of **1b + 2b** after 5h of reaction and dephosphorylation with alkaline phosphatase. For details see Method B.

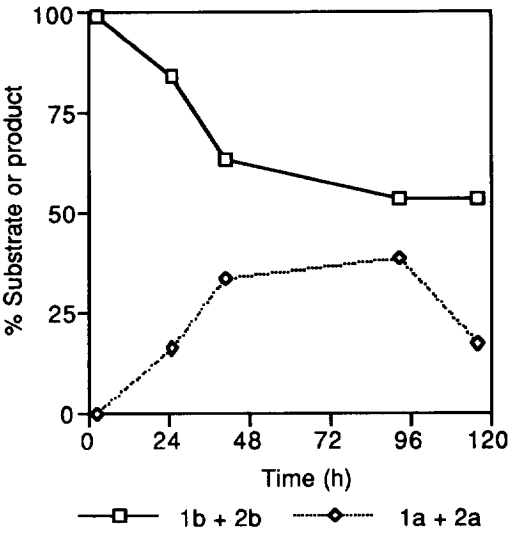


Figure 2. Kinetics of the dephosphorylation of **1b + 2b** catalyzed by 5'-nucleotidase from *Crotalus atrox* venom. For details see Method C.

enantiomers of nucleoside analogues can exhibit different biological effects with different enzymes or receptors.[2,8-10]

R,S-Adenallene 4'-phosphate **1b + 2b**, sodium salt. A mixture of R,S-adenallene[5] **1a + 2a** (162 mg, 0.8 mmol), 2-cyanoethyl phosphate[4] (1.6 mmol) and dicyclohexylcarbodiimide (0.99g, 4.8 mmol) in pyridine (15 mL) was stirred for 16 h at room temperature under N_2. Water (20 ml) was added and the mixture was evaporated. The residue was dissolved in 9 M NH_4OH (40 mL) and the solution was heated at 60°C for 1 h. After cooling, the solids were filtered off, the filtrate was evaporated and the residue was applied on a DEAE Sephadex A25 column which was eluted with water (800 mL) and then with a linear gradient water (2 L) - 0.4 M NH_4HCO_3 (2 L). The major peak was pooled, lyophilized and rechromatographed using 0.1 - 0.3 M NH_4HCO_3 gradient. The appropriate fractions were lyophilized, the residue was dissolved in water (30 mL) and the solution was stirred with Dowex 50 (Na^+, 30 g) for 15 min. The resin was filtered off and the filtrate was lyophilized to give phosphate **1b + 2b** (80 mg, 30%) as a sodium salt. Electrophoretic mobility[11] (pH 7) corresponded to that of AMP. HPLC[5] (Synchropak RP-P, 25 x 0.21 cm, water, flow rate 0.2 ml/min, detection at 262 nm, t_R 10.8 min. (97 %). UV max (pH 7) 259 nm (ε 12,000), 222 (ε 23,100). ^1H NMR (499.85 MHz, D_2O) ∂ 7.93 and 7.84 (2s, 2, H_2 and H_8), 6.96 (m, 1, $H_{1'}$), 6.18 (q, 1, $H_{3'}$), 4.37 (m, 2, $H_{4'}$); ^{13}C NMR (125.70 MHz) 196.63 ($C_{2'}$), 103.38 ($C_{3'}$, d, $^3J_{3',P}$ = 8.4 Hz), 93.45 ($C_{1'}$), 61.58 (d, $C_{4'}$, $^2J_{4',P}$ = 4.7 Hz), adenine: 154.80, 152.17, 146.95, 139.92, 117.83; ^{31}P NMR (121.49 MHz) 3.14.

Enzymatic Studies. A. Alkaline Phosphatase. Substrate **1b + 2b** or **3b + 4b** (3 μmol) was incubated with alkaline phosphatase (6 - 17 units) from chicken intestine mucosa in 0.1 M TRIS HCl (pH 8.6, 1 mL) at 37°C for 24 h. Paper electrophoresis (pH 7) showed quantitative degradation to adenallene **1a + 2a** or hypoxallene **3a +**

4a, mobilities relative to **1b** + **2b**: 1.26 (**3b** + **4b**), -0.05 (**1a** + **2a**, **3a** + **4a**).

B. AMP Deaminase. Phosphate **1b** + **2b** (3 μmol) was incubated with AMP deaminase from rabbit muscle (3 units) in 0.01 M sodium citrate (pH 6.5, 0.3 mL) at 25°C for 5 or 24 h. Paper electrophoresis after 24 h showed a complete conversion to R,S-hypoxallene 4'-phosphate **3b** + **4b**. The reaction after 5 h was quenched with ethanol (2 mL), the solution was evaporated and the residue was incubated with alkaline phosphatase for 16 h. The mixture was applied on a strip of Whatman 3MM paper and it was then subjected to electrophoresis at pH 7. A single UV-absorbing band moving toward the cathode was eluted with ethanol (20 mL). A 0.1 mL aliquot was subjected to HPLC on a Chiralcel CA-1 column[2] (25 x 0.49 cm, EtOH, 40°C, flow rate 0.5 mL/min, detection at 250 nm) to give **3a** + **4a** (t_R 8.22, 51%), **2a** (t_R 10.56, 25%) and **1a** (t_R 12.00, 24%, Figure 1).

C. 5'-Nucleotidase. Phosphate **1b** + **2b** (9 μmol) was incubated with 5'-nucleotidase from *Crotalus atrox* venom (7 units) in glycine buffer[7] (pH 9, 0.9 mL) at 37°C for a total of 116 h. The reaction was followed by HPLC on a Synchropak RP-P column[5] (5% MeCN in water, see compound **1b** + **2b** and Figure 2). In another experiment, the reaction mixture was incubated for 40 h whereupon it was subjected to paper electrophoresis as in Method B. The separated phosphate **1b** + **2b** and adenallene **1a** + **2a** were eluted with water and ethanol, respectively. An aliquot of the latter product was chromatographed as in Method B to give **2a** (37%) and **1a** (63%). The phosphate portion was digested with alkaline phosphatase and chromatographed on a Chiracel CA-1 column[2] to furnish **2a** (77%) and **1a** (23%).

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