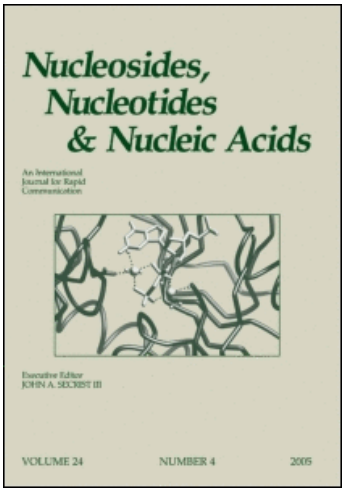


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# $O^6$-(4-Nitrophenyl)inosine and -Guanosine as Chromogenic Substrates for Adenosine Deaminase

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# $O^6$-(4-NITROPHENYL)INOSINE AND -GUANOSINE AS CHROMOGENIC SUBSTRATES FOR ADENOSINE DEAMINASE[1]

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**Abstract.** $O^6$-(4-Nitrophenyl)inosine (1a), $O^6$-(4-nitrophenyl)guanosine (1c) and $O^6$-(4-methylumbelliferonyl)inosine (2) were obtained by reaction of 6-chloro-9-(β-D-ribofuranosyl)purine (3a) or 2-amino-6-chloro-9-(β-D-ribofuranosyl)purine (3c) with sodium salts of 4-nitrophenol or 4-methylumbelliferone in N,N-dimethylformamide. Similarly, 6-chloro-9-(β-D-2,3-isopropylideneribofuranosyl)purine (3b) was transformed to 2',3'-O-isopropylidene-$O^6$-(4-nitrophenyl)inosine (1b). Deprotection of 1b with $CF_3COOH$ gave compound 1a and $O^6$-(4-nitrophenyl)hypoxanthine (4). Compounds 1a and 1c are substrates for adenosine deaminase releasing 4-nitrophenol which is readily detected visually or spectrophotometrically. Rate and extent of hydrolysis of 1a are significantly increased in the presence of purine nucleoside phosphorylase but xanthine oxidase has no influence. A potential fluorogenic analogue 2 is not a substrate for adenosine deaminase.

Adenosine deaminase (ADA, adenosine aminohydrolase, EC 3.5.4.4) is a catabolic enzyme converting adenosine to inosine[2]. It forms an important part of the purine salvage metabolic pathway and it is also crucial for regulation of adenine nucleotide metabolism[3]. A hereditary lack of ADA is associated with a severe combined immunodeficiency disease (SCID)[4]. The enzyme is also responsible for inactivation of biologically active adenosine analogues including the clinically effective antiviral agent vidarabine (araA)[5]. It is also an

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**Dedicated to Professor Yoshihisa Mizuno on the occassion of his 75th birthday.**

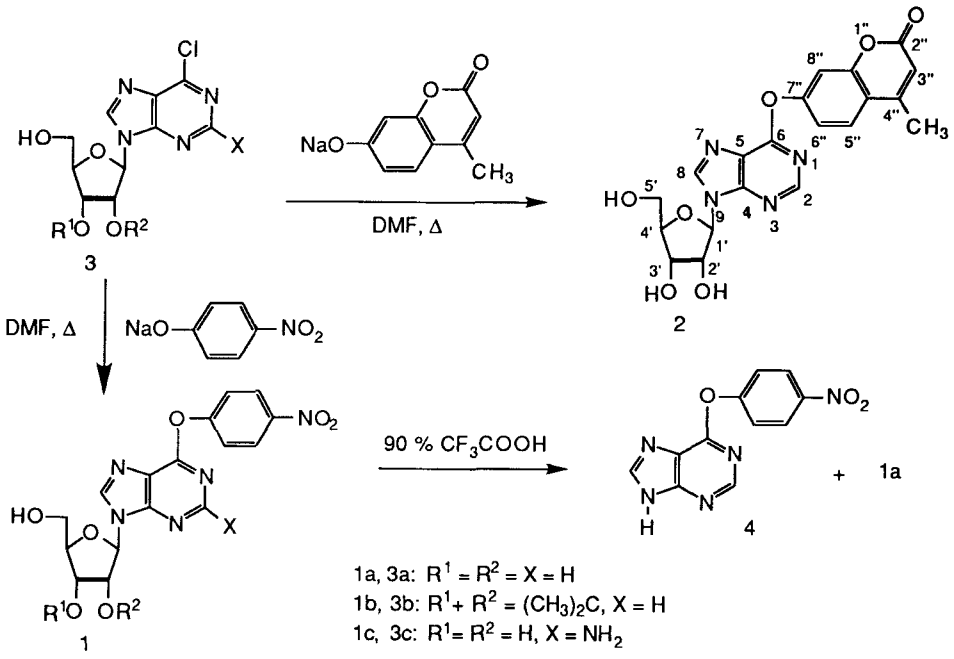
established malignancy marker[6]. ADA which in some respects resembles proteolytic enzymes[7] is capable of hydrolyzing nucleoside derivatives with a wide variety of leaving groups at the position 6 of purine ring[8-11].

Several assays are available for determination of ADA in biological materials. Thus, UV spectrophometry at 265 nm, using adenosine as a substrate, is frequently used for estimating the activity of purified enzyme[12]. Clinical methods make use of spectrophotometry or fluorometry based on ammonia released during deamination by coupling through glutamate dehydrogenase[13,14] or formation of indophenol[15].

Chromogenic substrates such as 4-nitrophenyl phosphate and related compounds, various 4-nitrophenyl carboxylic esters and glycosides are widely used and commercially available reagents for determination of many hydrolytic enzymes in biological materials[16]. The method is based on a release of yellow (UV max 400 nm) 4-nitrophenoxide ion during the enzymatically catalyzed hydrolysis at physiological pH. The sensitivity of assay can be increased by using fluorogenic substrates capable of releasing a fluorescent moiety, e. g., 4-methylumbelliferone. The application of these principles to ADA has been hampered by a lack of suitable chromogenic substrates. The present communication describes the synthesis of two nucleosides which release 4-nitrophenol by the action of ADA - $O^6$-(4-nitrophenyl)inosine (1a) and $O^6$-(4-nitrophenyl)guanosine (1c) as well as a potentially fluorogenic compound 2.



**Synthesis.** Chromogenic substrate 1a was prepared by two methods (Scheme 1). Nucleoside 3a was converted to the corresponding 2',3'-O-isopropylidene derivative 3b using 70 % $HClO_4$ in acetone (94 % yield). The reaction of 3b with sodium nitrophenoxide in N,N-dimethylformamide (DMF) at 50 - 55°C for 24 h gave intermediate 1b in 42 % yield. Deprotection of 1b in 90 % $CF_3CO_2H$ at room temperature for 30 min afforded compound 1a in 33 % yield. In addition, a depurination product, $O^6$-(4-nitrophenyl)hypoxanthine (4), was obtained (50 %). More directly, chloro nucleoside 3a was reacted with sodium 4-nitrophenoxide in DMF at 65 - 75°C for 25 h to furnish compound 1a in 19 % yield. In a similar fashion, 2-amino-6-chloro-9-(β-D-ribofuranosyl)purine (3c) and sodium 4-nitrophenoxide in DMF at 110 - 120°C for 20 h gave compound 1c ( 27 %). The re-




Scheme 1



action of 3a with sodium salt of 4-methylumbelliferone under the conditions described for the synthesis of 1a from 3a afforded compound 2 in 22 % yield.

**Biochemistry**. Compound 1a is hydrolyzed by ADA from calf intestine mucosa to give inosine and 4-nitrophenol in 0.05 M $Na_2HPO_4$ at pH 7.5. It is a relatively weak substrate requiring 1 - 2 mM concentration. Little activity was observed at 0.2 mM or below. The ADA-catalyzed hydrolysis was followed spectrophotometrically at 400 nm (Figure 1) and also by TLC in $CH_2Cl_2$ - MeOH (9 : 1) system. The reaction stopped at ca. 9 % conversion to 4-nitrophenol when the concentration of inosine reached 0.175 mM, i. e., the $K_i$ value of inosine[7,10] (0.16 - 0.184 mM). Therefore, we anticipated that removal of the latter nucleoside would increase the release of 4-nitrophenol from 1a. Indeed, addition of purine nucleoside phosphorylase (PNP, purine nucleoside: orthophosphate ribosyltransferase, EC 2.4.2.1), which is capable of cleaving inosine by phosphorolysis to hypoxanthine and α-D-ribose 1-phosphate[17], increased both the rate and conversion to 4-

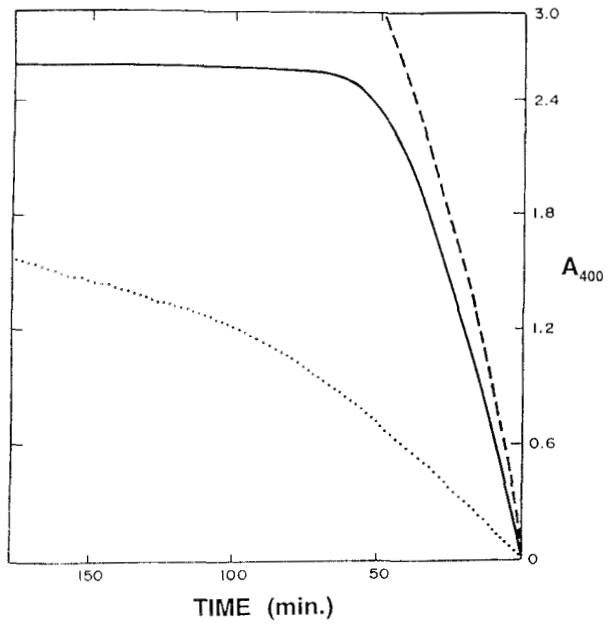



Figure 1. UV spectrophotometric traces of ADA-catalyzed hydrolysis of compounds 1a and 1c. For details see Experimental Section.



nitrophenol (44 %). It has to be noted that compound 1a is not a substrate for PNP. A simultaneous introduction of PNP and xanthine oxidase (XO, xanthine: oxygen oxidoreductase, EC 1.2.3.2 ), which oxidizes hypoxanthine to uric acid[18], into the assay system changed neither the reaction rate nor the extent of conversion. It should be noted that uric acid does not appear to interfere with PNP or ADA. Thus, coupled methods for quantitative assays of both enzymes based on conversion of hypoxanthine to uric acid are routinely used[17,19].

Using 2 mM solution of compound 1a at pH 7.5 it was possible to detect visually 0.02 unit ADA/mL in the absence of PNP (yellow coloration of 4-nitrophenoxide). This level corresponds to concentrations of ADA found in human serum[13]. Compound 1a is completely stable under the assay conditions without ADA. In fact, aqueous solutions (pH 7.5) are stable for many months at 0°C. Little ADA-catalyzed hydrolysis was observed

with heat-inactivated enzyme or when pancreatic ribonuclease was used as a non-specific protein. Most importantly, hydrolysis of 1a to 4-nitrophenol is completely inhibited by *erythro*-$N^9$-(2-hydroxy-3-nonyl)adenine (EHNA)[20]. The latter fact indicates that hydrolysis of 1a is a process catalyzed specifically by ADA. Compound 4 is completely resistant toward ADA as shown by a TLC assay. Guanine derivative 1c is also a substrate for ADA. However, as can be expected, the hydrolysis was slower than that of 1a and it effectively stopped at 17 % conversion to 4-nitrophenol after 2 days. Compound 1c is also completely stable under the assay conditions without ADA but it is less soluble in aqueous media. By contrast, umbelliferonyl derivative 2 is not a substrate for ADA as shown by a TLC assay. The final concentration of compound 1a was 2 mM in 0.05 M $Na_2PO_4$ (pH 7.5).

—— Compound 1a and ADA (20 units). Initial velocity V = 12.7 nmol/min. A constant $A_{400}$ value of 2.58 (8.7 % conversion) was reached after 100 min. The reaction in the presence of ADA deactivated by 1 min. heating at 100°C amounted to only 5 % of that catalyzed by active ADA. Pancreatic ribonuclease (20 units) did not catalyze any hydrolysis of 1a and 0.1 mM EHNA inhibited completely the hydrolysis.

- - - - Compound 1a, ADA (20 units) and PNP (2 units). V = 16.7 nmol/min. A constant $A_{400}$ value of 13.0 (43.6 % conversion) was reached after 5 h. The result obtained after addition of xanthine oxidase (2 units) was virtually the same (V = 16.9 nmol/min., conversion 43.7 % after 5 h).

····· Compound 1c (1 mM) and ADA (20 units). V = 3.1 nmol/min. A constant $A_{400}$ value of 2.56 (17.4 % conversion) was obtained after 2 days.

In multiple enzyme systems ADA was added as the last component.

However, it is not completely soluble at 1 mM level. Therefore, it cannot be excluded that the latter factor is responsible for a lack of substrate activity.

The assay procedure described herein should be especially suitable for a rapid detection of ADA in chromatographic eluates. A solution containing compound 1a (2 mM) and PNP in a phosphate buffer should be particularly advantageous.

## Experimental Section

**General Methods.** The $^1H$ NMR spectra were recorded by using a QE-300 instrument (General Electric) at 300 MHz in $CD_3SOCD_3$ with $Me_4Si$ as an internal reference unless

stated otherwise. Thin-layer chromatography (TLC) was performed on aluminum foils coated with Silica Gel 60 F254 (Merck). The solvent systems were as follows: $S_1$, $CHCl_3$ - MeOH (100 : 1.5), $S_2$, $CHCl_3$ - MeOH (37 : 3), $S_3$, $CH_2Cl_2$ - MeOH (9 : 1) and $S_4$, $CH_2Cl_2$ - MeOH (95 : 5). For chromatography on loose layers of silica gel see ref.[21] Melting points were determined on a Reichert Thermovar hot-stage apparatus unless stated otherwise and they are uncorrected. 4-Nitrophenol (Eastman Organic Chemicals, Rochester, New York) was recrystallized twice from water, mp. 112.5 - 113.5°C (Thomas-Hoover apparatus), $\varepsilon_{400}$ 17,600 (0.01 M NaOH), literature[22,23] $\varepsilon_{400}$ 18,320 and $\varepsilon_{401}$17,480, respectively; $\varepsilon_{400}$ 14,960 (0.05 M $Na_2HPO_4$, pH 7.5), $\varepsilon_{418}$ 11,830, literature[24] 11,500.

**Enzymes.** Adenosine deaminase (ADA) from calf intestine mucosa, type II or VIII, purine nucleoside phosphorylase (PNP) from bovine spleen, xanthine oxidase (XO) from buttermilk and ribonuclease from bovine pancreas were all products of Sigma Chemical Company, St. Louis, MO.

**6-Chloro-9-(β-D-2,3-O-isopropylideneribofuranosyl)purine (3b).** A mixture of 6-chloro-9-(β-D-ribofuranosyl)purine (3a, 3 g, 10.5 mmol), 70 % $HClO_4$ (0.5 mL) and acetone (200 mL) was stirred for 3 h at room temperature. A solid $Na_2CO_3$ (2 g, 19 mmol) was added and the stirring was continued for additional 3 h. The insoluble portion was filtered off and the filtrate was evaporated. The crude product was chromatographed on a column of silica gel (20 g) in $CHCl_3$ - MeOH (50 : 1) to give compound 3b (3.26 g, 94 %), mp. 160 - 162°C after recrystalization from methanol. Lit.[25,26] 154 - 157°C and 158 - 159°C, respectively.

**2',3'-O-Isopropylidene-$O^6$-(4-nitrophenyl)inosine (1b).** A mixture of compound 3b (1.0 g, 5.2 mmol) and sodium 4-nitrophenoxide (0.84 g, 5.2 mmol) was stirred in DMF (20 mL) at 50 - 55°C for 24 h. The solution was evaporated in vacuo (oil pump ) and the residue was partitioned between $CHCl_3$ (200 mL) and saturated aqueous $Na_2CO_3$ (100 mL). The organic phase was dried ($MgSO_4$) and it was evaporated. The crude product was chromatographed on a column of silica gel (10 g) using solvent system $S_1$ to give a TLC ($S_2$) homogeneous foam (0.56 g, 42 %). Compound 1b was recrystallized from

methanol (0.28 g, 21 %), mp. 157 -158°C (Thomas-Hoover apparatus). UV max (ethanol) 275 nm (ε 15,200). $^1$H NMR (100 MHz, $CDCl_3$ + $D_2O$) δ 8.51 (s, 1, $H_8$), 8.35 and 7.47 (2d, 4, J 9.1 Hz, 4-nitrophenyl), 8.19 (s, 1, $H_2$), 6.00 (d, 1, $H_{1'}$, $J_{1',2'}$ 4.5 Hz), 5.15 (m, 2, $H_{2'}$ and $H_{3'}$), 4.55 (apparent s, 1, $H_{4'}$), 3.89 (dq, 2, $H_{5'}$), 1.66 and 1.39 (2s, 6, $CH_3$). Anal. Calcd for $C_{19}H_{19}N_5O_7$: C, 53.14; H, 4.46; N, 16.31. Found: C, 52.92; H, 4.51; N, 16.33.

**$O^6$-(4-Nitrophenyl)inosine (1a). A. From 6-Chloro-9-(β-D-ribofuranosyl)purine (3a).** A mixture of compound 3a (0.87 g, 2.7 mmol) and sodium 4-nitrophenoxide (1.29 g, 8 mmol) in DMF (25 mL) was stirred at 65 - 75°C (bath temperature) for 25 h. After cooling, a fine precipitate of NaCl was filtered off, it was washed with DMF (10 mL) and the filtrate was evaporated in vacuo (oil pump, bath temperature 30°C). A solid orange residue was washed several times with $CH_2Cl_2$ (total 200 mL) and the solvent was removed. The remaining sirup was chromatographed on a single 3 mm thick loose layer[21] of silica gel 35 x 15 cm in solvent system $S_3$. A major UV-absorbing band was eluted with the same solvent system and the solvents were evaporated to give a sirup which crystallized after addition of $CH_2Cl_2$, 0.2 g (19 %) of compound 1a, mp. 182 - 184°C. Crystallization from water afforded an analytical sample, mp. 183 - 185°C, $R_F$ 0.5 ($S_2$). UV max (ethanol) 275 nm (ε 14,900); (0.05 M $Na_2HPO_4$, pH 7.5) 271 (ε 13,600). $^1$H NMR δ 8.81 (s, 1, $H_8$), 8.54 (s, 1, $H_2$), 8.34 and 7.61 (2d, 4, J 9.1 Hz, 4-nitrophenyl), 6.05 (d, 1, $H_{1'}$, $J_{1',2'}$ 5.5 Hz), 5.55 (d, 1, 2'-OH, $J_{OH,2'}$ 5.9 Hz), 5.27 (d, 1, 3'-OH, $J_{OH,3'}$ 4.8 Hz), 5.12 (t, 1, 5'-OH, $J_{OH,5'}$ 5.3 Hz), 4.63 (q, 1, $H_{2'}$), 4.19 (q, 1, $H_{3'}$), 3.99 (q, 1, $H_{4'}$), 3.68 and 3.60 (2m, 2, $H_{5'}$). Anal. Calcd for $C_{16}H_{15}N_5O_7$: C, 49.36; H, 3.88; N, 17.99. Found: C, 49.12; H, 4.01; N, 17.96.

**B. From 2',3'-O-Isopropylidene-$O^6$-(4-nitrophenyl)inosine (1b).** A solution of compound 1b (118 mg, 0.28 mmol) in 90 % $CF_3CO_2H$ (2 mL) was kept at room temperature for 2 h whereupon it was lyophilized. The residue was dissolved in water (2 mL) and it was lyophilized again. Methanol (2 mL) was then added and $O^6$-(4-nitrophenyl)hypoxanthine (4) was filtered off, 26 mg (36 %), mp. >320°C, $R_F$ 0.6 ($S_3$). UV max

(ethanol) 275 nm (ε 13,900). $^1$H NMR δ 8.56 and 8.49 (2s, 2, $H_2$ and $H_8$), 8.36 and 7.61 (m, 4, J 9.1 Hz, 4-nitrophenyl). Anal. Calcd for $C_{11}H_7N_5O_3.1/6\ H_2O$: C, 50.77; H, 2.84; N, 26.92. Found: C, 51.05; H, 3.15; N, 26.54.

The filtrate was evaporated and the residue was chromatographed on a single 2 mm thick 20 x 20 cm layer of silica gel GF (Uniplate$^{TM}$, Newark, Delaware) in solvent system $S_3$ (triple development). Three bands were obtained in order of decreasing mobility: Compound 1b (weak), 4 and 1a. Elution gave 4 (10 mg, 14 %) and 1a (33 mg, 33 %). The latter was identical with a sample prepared by method A.

**$O^6$-(4-Nitrophenyl)guanosine (1c).** The procedure for synthesis of compound 1a (method A) was followed using 2-amino-6-chloro-9-(β-D-ribofuranosyl)purine (3c, 0.91 g, 3 mmol), sodium 4-nitrophenoxide (0.58 g, 3.6 mmol) in DMF (25 mL) and 110 - 120°C for 20 h. The precipitated NaCl was filtered off and the filtrate was evaporated. The crude product was chromatographed on two loose layers[21] of silica gel (see compound 1a, method A). After a single development, the upper part of the layer containing 4-nitrophenol was removed, the layer was restored with a fresh silica gel and it was developed again in the same solvent. The major UV-absorbing band was eluted to give 0.47 g (39 %) of 1c, mp. 200 - 225°C, containing according to TLC ($S_3$) a trace of a tailing component. Crystallization from water (40 mL) afforded pure 1c, 0.33 g (27 %), mp. 210 - 211°C, $R_F$ 0.35 ($S_3$). UV max (ethanol) 290 nm (ε 14,800), 252 (ε 13,700 ); (pH 7.5) 291 (ε 16,400), 252 (ε 12,700), 213 (ε 28,000). $^1$H NMR δ 8.31 and 7.56 (2d, 4, J 9.1 Hz, 4-nitrophenyl), 8.28 (s, 1, $H_2$), 6.60 (s, 2, $NH_2$), 5.84 (d, 1, $H_{1'}$, $J_{1',2'}$ 5.9 Hz), 5.44 (bd, 1, 2'-OH), 5.16 (bs, 1, 3'-OH), 5.07 (bs, 1, 5'-OH), 4.51 (apparent d, 1, $H_{2'}$), 4.13 (t, 1, $H_{3'}$), 3.92 (q, 1, $H_{4'}$), 3.65 and 3.55 (2dd, 2, $H_{5'}$). Anal. Calcd for $C_{16}H_{16}N_6O_7.1/2\ H_2O$: C, 46.49; H, 4.15; N, 20.33. Found: C, 46.57; H, 4.14; N, 20.43.

**$O^6$-(4-Methylumbelliferonyl)inosine (2).** A method described for compound 1a was employed on the same scale using compound 3a and sodium salt of 4-methylumbelliferone as reactants. After filtration of NaCl the clear solution was evaporated, the resultant

foam was mixed with silica gel (10 g) and $CH_2Cl_2$ - MeOH (1 : 1, 30 mL). The mixture was evaporated to dryness and the solid residue was put on the top of a silica gel column (40 g, 5.5 x 5 cm) which was eluted with $CH_2Cl_2$ (200 mL), solvent system $S_4$ (1.3 L) and $S_3$ (500 mL). The appropriate fractions were evaporated to give 0.35 g (27 %) of compound 2, mp. 215 - 218°C. Crystallization from water (0.1 g/30 mL) afforded an analytical sample (22 %), mp. 217 - 218°C, $R_F$ 0.7 ($S_3$). UV max (ethanol) 313 nm (ε 12,700), 273 (ε 14,100), 210 (ε 34,700), shoulder 260 (ε 13, 300); (pH 7.5) 312 (ε 13,200), 261 (ε 14,200), 209 (ε 35,400), shoulder 274 (ε 13,900). $^1$H NMR δ 8.80 (s, 1, $H_8$), 8.52 (s, 1, $H_2$), 7.86 (d, 1, $H_{5''}$, $J_{5'',6''}$ 8.7 Hz), 7.47 (d, 1, $H_{8''}$, $J_{8'',6''}$ 2.3 Hz), 7.35 (dd, 1, $H_{6''}$, $J_{6'',5''}$ 8.7 Hz, $J_{6'',8''}$ 2.3 Hz), 6.39 (d, 1, $H_{3''}$, $J_{3'',Me}$ 1.1Hz), 6.05 (d, 1, $H_{1'}$, $J_{1',2'}$ 5.6 Hz), 5.56 (d, 2, 2'-OH, $J_{OH,2'}$ 6 Hz), 5.27 (d, 1, 3'-OH, $J_{OH,3'}$ 5 Hz), 5.14 (t, 1, 5'-OH, $J_{OH,5'}$ 5.5 - 5.6 Hz), 4.63 (q, 1, $H_{2'}$), 4.20 (q, 1, $H_{3'}$), 4.00 (q, 1, $H_{4'}$), 3.71 and 3.59 (dt and dq, 2, $H_{5'}$), 2.46 (d, 3, Me, $J_{Me,3''}$ 1.1 Hz). Anal. Calcd for $C_{20}H_{18}N_4O_7$: C, 56.34; H, 4.26; N, 13.14. Found: C, 56.17; H, 4.42; N, 12.99.

**ADA Assays. A. Spectrophotometric Method.** Substrate (1 - 2 mM) in 0.05 M $Na_2HPO_4$ (pH 7.5) was placed in a spectrophotometric cell. The final volume was 3.1 mL. The reaction was started by adding ADA (Type VIII) and it was followed continuously at 400 nm using a Perkin-Elmer Lambda 5 UV/VIS spectrophotometer in a time drive mode. At higher conversions the hydrolysis was monitored at 450 nm. A factor of 5.15 was used to convert $A_{450}$ to $A_{400}$. The results are summarized in Figure 1.

**B. TLC Method**[27]. Substrate (2.3 μmol) was magnetically stirred with ADA (Type II, 0.4 units) in 0.05 M $Na_2HPO_4$ (pH 7.6, 0.4 mL) at room temperature. Aliquots were periodically removed and analyzed by TLC in solvent system $S_3$. Authentic samples of substrates and reaction products were run simultaneously on the same plate.

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