BIOORGANIC CHEMISTRY 3, 367–376 (1974)

Reaction of the Ribose Moiety of Adenosine and AMP with Periodate and Carboxylic Acid Hydrazides

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Received December 18, 1973

The dialdehyde (II–V) generated by periodate oxidation of the ribose moiety in adenosine or AMP reacts readily with carboxylic acid hydrazides yielding morpholine derivatives (VI, VII, VIII) which are stable over a wide range of pH and temperature. No side reactions have been observed. This reaction will allow introduction of various substituents into the 3′ end of RNAs, and the resulting modifications would permit investigations on the structure and function of such RNAs.

INTRODUCTION

Modification of biopolymers plays an important role in the investigation of their chemical, physical, and biochemical properties. One of the most specific reactions of RNA molecules is periodate oxidation, by which a reactive dialdehyde moiety is generated at the 3′-terminus (I → II–V), which can then serve as a site for further reactions, e.g., degradation of the 3′-end nucleoside (*1*), introduction of spectroscopic labels (*2*), binding to a polymer support (*3–5*), and preparation of molecules with two primary hydroxyl groups by subsequent borohydride reduction (*6–8*). Despite the fact that the reaction of periodate-oxidized ribonucleotides with amines and hydrazines has frequently been used in biochemical studies (*9–14*), the mechanisms of these reactions as well as the composition of the reaction products, are still a matter of discussion (*12, 13*). Primary amines react to form aldimines which are extremely unstable with respect to elimination of the 5′-phosphate group. Similarly, the condensation products formed with hydrazines and semicarbazides (*14–16*) are unstable. In 1965 isonicotinic acid hydrazide was allowed to react with periodate-oxidized nucleosides (*17*), and the reaction products were analyzed by uv spectroscopy and combustion analyses; however, these analytical data were insufficient to establish a unique structure. We wish to report that in the case of the model compounds adenosine$_{oxi}$ and AMP$_{oxi}$, isonicotinic acid hydrazide, Girard reagent P, and benzoic acid hydrazide are convenient reagents for forming unique, stable products (VI–VIII). While this work was in progress, the reaction of periodate-oxidized uridine with benzoic acid hydrazide was investigated by others (*18*); in general, the conclusions drawn regarding the structure of the reaction product are in agreement with ours.

[1] *Abbreviations:* Adenosine$_{oxi}$ and AMP$_{oxi}$: periodate oxidation products of adenosine and AMP, respectively (II–V).

RESULTS AND DISCUSSION

Stability and Structure of the Periodate Oxidation Products of Adenosine and AMP (II–V)

The oxidation of *cis*-diol groups with periodate is a second-order reaction which proceeds at room temperature at a reasonable rate (*19*). Decomposition of the dialdehyde formed and formation of by-products can be suppressed when the reaction is carried out in the dark (*20*). The stability of the dialdehyde obtained by the oxidation of nucleotides (e.g., I → II, R=$(HO)_2PO$) is dependent upon the temperature and pH value of the solution. AMP_{oxi} (II, R=$(HO)_2PO$) decomposes slowly with formation of an unknown intermediate which is transformed further into $adenosine_{oxi}$ (II, R=H). The

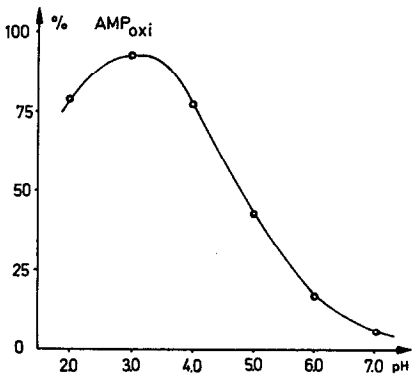

FIG. 1. Stability of AMP_{oxi} (II–V, R=$(HO)_2PO$) as a function of pH. Ten microliters of a freshly prepared 10^{-2} *M* AMP_{oxi} solution and 10 μl 10^{-1} *M* buffer (for pH 2.0: Na–citrate/HCl (Sørensen), for pH 3.0–7.0: Na_2HPO_4/citric acid (McIlvain), which did not contain amines) were incubated for 48 hr at 37°C. The mixture was analyzed by tlc, and the AMP_{oxi} spot eluted with 2 ml H_2O and determined by measuring the optical density at 260 nm.

half-lives of the decomposition at 4, 20, and 37°C (pH 7.0) are 17 days, 45 and 15 hr, respectively. The influence of the pH value on the stability of AMP_{oxi} is revealed in Fig. 1. Stability is optimal in the region of pH 3.0, while at lower or higher pH, one observes enhanced irreversible decomposition of the phosphodialdehyde.

The rate of decomposition of $adenosine_{oxi}$ (II–V, R=H) is not as high as was previously assumed (*21*). It can be recrystallized from hot water without detectable decomposition, and even be dissolved in 1 *M* HCl at room temperature. The low solubility in water stands in contrast to the high solubility in hydrochloric acid. The enhanced hydrophobicity of $adenosine_{oxi}$ indicates a structure markedly different from that of a free dialdehyde (*22*). Combustion analyses provide the theoretical values for the dialdehyde (II, R=H). However, neither a C=O group absorption band in the expected region of 1725 cm^{-1} nor frequencies at 2855 and 2740 cm^{-1} due to aldehydic hydrogen are present in the ir spectrum of this substance. On the other hand, the C—O—C band around 1100 cm^{-1} which is characteristic for polymerized aldehydes (*23*), e.g., paraformaldehyde, can be identified. The downfield shift of H_2, and H_3, in the nmr spectrum is not as large as would be expected, since a free dialdehyde normally appears at 9–10 ppm. At

I
I: R = H, adenosine
I: R = (HO)2PO, AMP
IO4⊖
II
II–V: R = H, adenosineoxi
II–V: R = (HO)2PO, AMPoxi
H2O
H2O
III
IV
(HO)2HC
CH(OH)2
–xH2O
V
II–V:
+ isonicotinic
acid hydrazide
VI
II–V
+ Girard P
reagent
VII
II–V
+ benzoic acid
hydrazide
VIII

room temperature, one obtains a spectrum with broad signals similar to that of a polymeric aldehyde with asymmetric centers, e.g., glutaraldehyde (*22*). Probably the adenosine$_{oxi}$ molecules form oligo- and polyribooxynucleosides or -nucleotides with concomitant release of water, even in concentrated aqueous solution (II$\rightleftharpoons$ III/IV $\rightleftharpoons$ V); compound V probably exists also in the solid phase. Since AMP$_{oxi}$ is very unstable with respect to β-elimination and is not crystalline (e.g., *14*), it cannot be investigated as successfully as can adenosine$_{oxi}$. However, from nmr data, being in general identical with those of adenosine$_{oxi}$ but downfield shifted by about 1.0–1.5 ppm (p. 375), and from the characteristic "elongated spots" (e.g., *14*) on chromatography an analogous hydration and dehydration scheme can be entertained.

Formation and Stability of the Condensation Products of Adenosine$_{oxi}$ or AMP$_{oxi}$ and Carboxylic Acid Hydrazides (II–V → VI–VIII)

The reaction of periodate-oxidized nucleosides and nucleotides with carboxylic acid hydrazides proceeds with 1 : 1 stoichiometry (II–V → VI–VIII) (*17*, *18*). On the one hand, the nucleophilicity of hydrazides is sufficient for a rapid reaction; on the other hand their limited basicity (*24*) tends to prevent labilization and release of the phosphate group by β-elimination (*12*). Even when using a 100-fold excess of the hydrazide, no

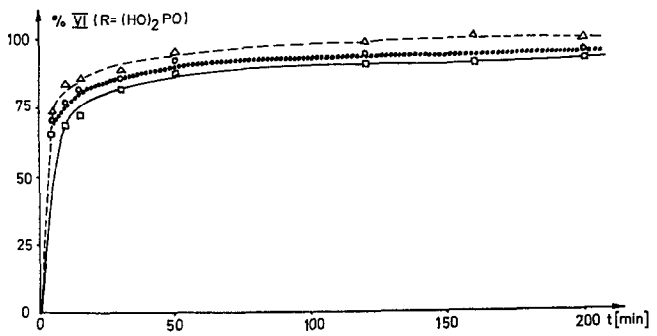

FIG. 2. Kinetics of the reaction of AMP$_{oxi}$ with isonicotinic acid hydrazide to give VI (R=(HO)$_2$PO) (23°C). One hundred microliters 10^{-2} *M* AMP$_{oxi}$ were incubated with 100 μl 10^{-2} *M* isonicotinic acid hydrazide and 100 μl 10^{-1} *M* Teorell–Stenhagen buffer pH 4.9 (△--△), 6.9 (○·····○) and 8.8 (□–□). Samples of 10 μl were withdrawn and analyzed by tlc; VI (R=(HO)$_2$PO) was eluted with 2 ml H_2O and determined by measuring the optical density at 260 nm.

β-elimination takes place in the reaction of AMP$_{oxi}$. The strong basicity of amines and hydrazines and their ability to accept a proton is the main reason for β-elimination of the 5′-phosphate from the intermediate Schiff base (*12*, *14*).

The pH-dependence of the formation and stability of the condensation products was determined in the range of pH 3.5–9.8 as well as in 0.1 *N* HCl and 0.1 *N* NaOH. In strong alkaline and acidic solution no condensation occurs. No detectable decomposition of the products was observed between pH 3.5 and 9.8 after 12 hr at 24°C. The reaction velocity is higher at pH 4.9 than under neutral or slightly alkaline conditions (Fig. 2).

The yields with all hydrazides used were quantitative. In the reactions with hydrazides described here, no side reactions with adenine, guanine, cytosine (*25*), and uracil were observed under the conditions given. Hydrazide derivatives of adenosine$_{oxi}$ (VI–VIII,

R=H) can be recrystallized without decomposition, and the products are stable for several months.

Structure of the Reaction Products VI–VIII of Adenosine$_{oxi}$ or AMP$_{oxi}$ and Carboxylic Acid Hydrazides

The structures proposed for the reaction products of adenosine$_{oxi}$ or AMP$_{oxi}$ with hydrazides (VI–VIII) are based upon combustion analyses as well as uv, ir, nmr, and mass spectra.

The combustion analyses, melting points, and R_f values of the reaction products are given in Table 1. Ultraviolet difference spectra of VI (R=$(HO)_2PO$) with AMP$_{oxi}$ and isonicotinic acid hydrazide show that the chromophoric systems are not changed

TABLE 1

YIELDS, MELTING POINTS, AND COMBUSTION ANALYSES

Substance	Emp. formula (M_r)	Yield (%)	mp (°C)	Combustion analysis C Calcd; found	H Calcd; found	N Calcd; found
VI (R=H)	$C_{16}H_{18}O_5N_8$	70	220	47.8	4.48	27.85
	(402.37)		(dec)	47.62	4.69	27.81
VII (R=H)	$C_{17}H_{21}O_5N_8Cl$	Quant.	210	45.0	4.65	24.8
	(452.87)		(dec)	45.17	4.5	25.08
VIII (R=H)	$C_{17}H_{19}O_5N_7$	85	180	50.9	4.8	24.45
	(401.37)		(dec)	50.82	4.85	24.43

(Table 1). In the mass spectra the molecular peak was not identifiable, but the ions generated by the release of 2 moles of water were observed. Due to the lability of quaternary salts, no interpretable mass spectrum was obtained for VII.

The absorption bands (Table 2) of amide I and amide II appear at 1675 and 1850 cm^{-1}, respectively. The —CO—NH— group in polar solvents has the *trans* configuration

TABLE 2

INFRARED SPECTRA OF HYDRAZIDES OF ADENOSINE$_{oxi}$ $\tilde{\nu}$ [cm^{-1}]:

Substance	Amide carbonyl stretch (C=O)	Sec. amide stretch (NH)	Amide I (CO—NH)	Amide II (CO—NH)	H | —N—C— || O (*trans*)
VI (R=H)	1650	3335, 3075	1675	1580	1540
VII (R=H)	1640	3340, 3360	1680	1575	1545
VIII (R=H)	1645	3330, 3070	1680	1580	1535

(1540 cm^{-1}; for comparison: *cis*-1470 cm^{-1}), which is in agreement with the nmr investigations of Exner (*24*) on comparable molecular systems. These results show that the NH group, adjacent to the carbonyl group, did not change during the course of the reaction. The ir and nmr spectra give no indication of the presence of a free aldehyde group, proposed previously for the reaction of only 1 mole of hydrazide with the oxidized ribose moiety (*26*). The hydrogens of the newly formed morpholine ring give broad multiplets in the nmr spectra (Table 3), due to the presence of two new asymmetric

TABLE 3

NUCLEAR MAGNETIC RESONANCE DATA OF HYDRAZIDES OF ADENOSINE$_{oxi}$

Protons[a]	VI (R=H)	VII (R=H)	VIII (R=H)
H8	8.3[b] (m) (s)	8.3[b] (m) (s)	8.3[b] (m) (s)
H_2	8.12 (s)	8.21 (s)	8.22 (s)
NH_2	7.3 (s)	7.37 (s)	7.3 (s)
—CO—NH—	9.6/9.8 (2s), 1H	9.2/9.4 (2s), 1H	9.3/9.5 (2s), 1H
$H_{1'}$	6.1 (m), 1H	6.13 (m), 1H	6.1 (m), 1H
$H_{2'}$, $H_{3'}$	5.6–5.32 (m), 2H	5.8–5.6 (m), 2H	5.7–5.3 (m), 2H
$H_{4'}$	4.07 (m) 3H	4.1 (m) 3H	4.12 (m) 3H
$H_{5'}$	3.65 (m)	3.7 (m)	3.7 (m)
Residue of the hydrazide	H_2, H_6 (pyridine) 8.7 (d), 2H, J = 5 Hz H_3, H_5 (pyridine) 7.77 (d), 2H, J = 5 Hz	$—CH_2—\overset{+}{N}=$4.9 (s), 2H H_2, H_6 (pyridine) 9.3 (d), 2H H_3, H_5 (pyridine) 8.19 (m), 2H H_4 (pyridine) 8.63 (m), 1H	H_3, H_4, H_5 (benzene) 7.5 (m), 3H H_2, H_6 (benzene) 7.9 (m), 2H

[a] Numbering see formula VI.
[b] (m) at 25°C, (s) at 60°C.

centers ($C_{2'}$), ($C_{3'}$) and the fact that two positions of the N atom are possible in the heterocycles. For the NH proton of the hydrazide group we observed two singlets. The protons of the sugar moiety were determined by D_2O exchange and decoupling experiments. When the spectrum was recorded at room temperature, a doublet for the H_8 of the adenine ring was observed. Nuclear magnetic resonance experiments at various temperatures and subsequent model-building studies showed that the rotation of the purine ring around the anomeric center of the adenosine$_{oxi}$-hydrazide derivative is hindered.

Hydrolysis of the 5′-phosphate group of AMP$_{oxi}$ -hydrazides led to products which were identical with adenosine$_{oxi}$-hydrazides (Table 4). This result argues against the proposal of Guthrie (*27*), who suggested that the 5′-OH group is involved in a hemiacetal ring formation with the 2′-aldehyde group. In this case only the 3′-aldehyde would be free for the reaction with hydrazides, thus explaining the 1:1 stoichiometry of the reaction. Since AMP$_{oxi}$ with a blocked 5′-OH group led to the same product as adenosine$_{oxi}$, only the interpretation given in formula (VI–VIII) is possible.

TABLE 4

ULTRAVIOLET DATA AND R_f VALUES OF HYDRAZIDES OF ADENOSINE$_{oxI}$ AND AMP$_{oxI}$

Substance	UV-Data[a]	Thin-layer chromatography						Thin-layer electrophoresis	
	λ_{max} [nm]	Cellulose R_f	PEI Cellulose R_f	Silicagel R_f	Cel 300 CM[b] R_f	Cel 300 DEAE[b] R_f	Cel 300 ECTEOLA[b] R_f	Cellulose[c] (related to corresponding hydrazide = 1.0(–))	Cellulose[d] (related to AMP$_{oxI}$ = 1.0(+))
VI (R=(HO)$_2$PO)	258	0.73	0.06	0.68	0.89	0.53	0.80	0.15 (–)	0.65 (+)
VI (R=H)[e]	258	0.52	0.58	0.28	0.65	0.69	0.55	0.40 (–)	0.57 (–)
VI (R=H)[f]	258[g]	0.51	0.59	0.28	0.64	0.67	0.55	0.40 (–)	0.56 (–)
Isonicotinic acid hydrazide	263	0.73	0.78	0.42					
VII (R=(HO)$_2$PO)	259	0.65	0.38	0.15	0.85		0.86	0.23 (–)	0.23 (–)
VII (R=H)[e]	259	0.58	0.53	0.20	0.60		0.64	0.39 (–)	1.28 (–)
VII (R=H)[f]	259[g]	0.58	0.54	0.21	0.59		0.64	0.38 (–)	1.29 (–)
Girard P reagent	259	0.92	0.89	0.26					
VIII (R=(HO)$_2$PO)	256	0.46	0.10	0.72	0.86	0.42	0.78	0.06 (+)	0.70 (+)
VIII (R=H)[e]	256	0.55	0.50	0.55	0.65	0.58	0.55	0.33 (–)	0.50 (–)
VIII (R=H)[f]	256[g]	0.56	0.52	0.54	0.65	0.59	0.55	0.34 (–)	0.51 (–)
Benzoic acid hydrazide	226	0.75		0.56					

[a] In 0.1 *M* K-phosphate, pH 7.0.
[b] Purchased from Macherey & Nagel, Düren (Germany).
[c] In 0.1 *M* Na-formiate, pH 3.5; 600 V, 20 mA.
[d] In 0.1 *M* Sörensen citrate buffer; 600 V, 42 mA.
[e] Prepared from VI, VII, VIII, respectively, (R=(HO)$_2$PO) by treatment with alkaline phosphatase.
[f] Prepared from II–V (R=H) and corresponding hydrazide.
[g] ε_{max} [cm^2/mole]·10^3: 20.0, 20.5, 20.4, respectively.

CONCLUSION

Investigation of the reaction between periodate-oxidized nucleosides and nucleotides with hydrazides indicates that the introduction of various molecules into the 3′-terminus of RNAs should be possible by this method. Optimum reaction conditions appear to be pH 4.5–5.0 at 4°C. Thus, e.g., introduction of spectroscopic labels, heavy atom derivatives, and reactive groups will allow spectroscopic studies, X-ray structural analysis, and affinity labeling experiments, respectively, with various RNA species as tRNA, rRNA, and mRNA.

EXPERIMENTAL

Adenosine, AMP (adenosine-5′-phosphate), benzoic acid hydrazide, isonicotinic acid hydrazide, and Girard reagent P, (1-carbazoylmethyl)-pyridinium chloride were commercial products. Alkaline phosphatase from *E. coli* (EC 3.1.3.1) was a product of Boehringer, Mannheim, Germany.

Nuclear magnetic resonance spectra were measured on a Bruker HX 60 spectrometer using dimethylsulfoxide d-6 (DMSO-d-6) as a solvent and tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained on an Atlas-Varian-MAT CH-4 spectrometer. Infrared spectra were measured in KBr pellets using a Perkin–Elmer-Infracord device. Zeiss PMQ II and UNICAM SP 1800 spectrophotometers were used for measuring the uv spectra.

Thin-layer chromatography (tlc) was performed on plates of silica gel (Woelm, Eschwege, Germany), cellulose (Merck, Darmstadt, Germany), and polyethyleneimine cellulose (Schleicher & Schüll, Dassel, Germany) with 0.25 *M* aqueous LiCl as a solvent.

Preparation of Adenosine$_{oxi}$ (*II–V, R=H*)

Ten mmoles adenosine were treated with 10 mmole sodium metaperiodate in 100 ml aqueous solution, with stirring in the dark and cooling with ice water. After 1 hr the solution was concentrated to 50 ml by evaporation in vacuo at 30°C. After standing for 12 hr at 4°C the crystalline product, which was homogeneous by tlc, was filtered off, washed with cold water, and dried over silica gel in vacuo (12 mm Hg). Yield 2.4 g (90%): mp 210°C (dec).

Combustion anal. Calcd for $C_{10}H_{11}O_4N_5 \cdot H_2O$ (283.26): C, 42.2; H, 4.63; N, 24.7. Found: C, 42.24; H, 4.83; N, 24.52. uv (0.1 *M* potassium phosphate buffer, pH 7) $\lambda_{max} = 258$ nm, $\varepsilon_{max} = 14\,900$ cm^2/mole. ir: —C—O—C— ether band 1110 cm^{-1}, characteristic vibrations of aldehydes at 1725 cm^{-1} (C=O) as well as at 2740 cm^{-1} and 2855 cm^{-1} (—CO—H) were not observed. MS: 265 (4%) M^+, 247 (2%) $M^+ - H_2O$, 236 (5%) $M^+ - CHO$, 218 (10%) $M^+ - CHO - H_2O$, 191 (4%) B—CO—$\overset{+}{C}$HO$^+$ (McLafferty rearrangement from M^+), 177 (35%) B—CH=$\overset{+}{C}$HOH, 164 (100%) B—CH—$\overset{+}{O}$H, 148 (45%) B^+=CH_2, 136 (55%) BH^+, 135 (62%) B^+, 119 (15%) $B^+ - NH_2$, 108 (41%) 135-HCN, 81 (15%) 108-HCN (*m/e*). NMR (DMSO, TMS, 80°C; in ppm): 8.28 (s/1H) H_8, 8.19 (s/1H) H_2, 6.8 (s/2H) NH_2, 6.5–5.1 (m/3H) $H_{1'}$, $H_{2'}$, $H_{3'}$, 4.7–3.5 (m/3H) $H_{4'}$, $2H_{5'}$.

After 3 days' drying at 80°C, over P_2O_5 in vacuo (0.01 mm Hg) the water of hydration was lost: mp 210°C (dec).

Combustion anal. Calcd for $C_{10}H_{11}O_4N_5$ (265.24) C, 45.3; H, 4.15; N, 26.4. Found: C, 45.26; H, 4.15; N, 26.48. The uv, ir, ms, and nmr data were identical with those obtained for the adenosine $_{oxi}$ monohydrate.

Preparation of Hydrazides of Adenosine$_{oxi}$ (VI–VIII, R=H)

(a) *Reaction of adenosine$_{oxi}$ with isonicotinic acid hydrazide (II–V → VI, R=H) and benzoic acid hydrazide (II–V → VIII, R=H).* Ten mmoles adenosine were reacted with 10 mmoles sodium metaperiodate in 100 ml water. After 1 hr an aqueous solution of 10 mmole carboxylic acid hydrazide was added and left at 4°C overnight. The white crystalline product was filtered off and recrystallized from water and dried at 80°C over P_2O_5 in vacuo (0.01 mm Hg).

(b) *Reaction of adenosine$_{oxi}$ with Girard P reagent (II–V → VII, R=H).* A suspension of 1 mmole adenosine$_{oxi}$ monohydrate in 25 ml H_2O was treated with 1 mmole Girard P reagent at 37°C for 3.5 hr. At the end of this time, adenosine$_{oxi}$ had completely dissolved. The reaction mixture was evaporated under reduced pressure to dryness. The residue was recrystallized from ethanol/water. The crystalline, hygroscopic product was dried at 80°C over P_2O_5 in vacuo (0.01 mm Hg).

MS: *VI (R=H)*: 366 (1%) M^+–$2H_2O$, 348 (2%) 366-H_2O, 136 (26%) BH^+, 135 (100%) B^+, 108 (74%) 135-HCN, 81 (30%) 108-HCN, 122 (99%) pyr–$CONH_2^+$, 106 (94%) 122-NH_2, 78 (87%) 106-CO, 51 (51%) 78-HCN, 231 (4%) 366-B, 213 (6%) 348-B, 244 (3%) 366–122, 242 (3%) 348–106, 163 (12%) B–CHO^+ (*m/e*).

VII (R=H): 304 (3%), 281 (3%), 207 (7%), 148 (13%), 135 (92%) B^+, 109 (35%), 79 (100%) Pyr^+, 52 (100%) $C_4H_4^+$ (*m/e*).

VIII (R=H): 365 (2%) M^+–$2H_2O$, 347 (4%) 365-H_2O, 230 (3%) 365-B, 244 (4%) 365-C_6H_5—CONH, 136 (33%) BH^+, 135 (100%) B^+, 121 (63%) C_6H_5—$CONH^+$, 122 (39%) C_6H_5—$CONH_2$, 105 (100%) C_6H_5—CO^+, 77 (100%) C_6H_5 (*m/e*).

Condensation of AMP$_{oxi}$ with Hydrazides (II–V → VI–VIII, R=(HO)$_2$PO)

A $2 \cdot 10^{-2}$ *M* solution of AMP was treated with an equivalent amount of a $2 \cdot 10^{-2}$ *M* aqueous solution of sodium metaperiodate. The reaction mixture was left at 4°C for 30 min. Then a drop of 2,3-butanediol was added in order to destroy the excess of periodate. An equivalent amount of a $2 \cdot 10^{-2}$ *M* solution of the hydrazide in Teorell–Stenhagen buffer pH 4.9 (up to a concentration of 0.1 *M*) was then added and the reaction mixture left at room temperature for 2 hr. The reaction was followed by thin-layer chromatography (Table 4). For determination of the stoichiometry of the reaction, various ratios of hydrazide/AMP$_{oxi}$ were used (see Results).

Dephosphorylation of Hydrazides of AMP$_{oxi}$ (VI–VIII, R=(HO)$_2$PO) with Alkaline Phosphatase

To 50 μl of 5×10^{-3} *M* AMP$_{oxi}$ hydrazide solution were added 10 μl 1 *M* ammonium acetate buffer, pH 8.8, and 10 μl alkaline phosphatase. The solution was incubated at 37°C for 40 min. The products were characterized by thin-layer chromatography.

Nuclear magnetic resonance data of AMP$_{oxi}$ (in D_2O at 25°C, TMS, ppm). 5.1–4.3 (m/3H) $2H_{5'}$,$H_{4'}$; 6.6–5.9 (m/3H) $H_{1'}$, $H_{2'}$, $H_{3'}$; 8.75–9.2 (m/2H) H_2, H_8.

ACKNOWLEDGMENTS

We gratefully acknowledge discussion of the manuscript with Dr. D. Gauss.

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