Alkylation of Nucleosides and Nucleotides by Dehydroretronecine; Characterization of Covalent Adducts by Liquid Secondary Ion Mass Spectrometry[⊥]

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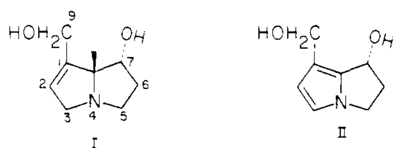
Abstract: In vitro reaction of dehydroretronecine with the nucleosides guanosine, adenosine, deoxyadenosine, uridine, and deoxythymidine and the nucleotides deoxyguanosine 5′-monophosphate, deoxyadenosine 5′-monophosphate, deoxythymidine 5′-monophosphate, and deoxyuridine 5′-monophosphate under mild basic conditions results in monoalkylated covalent adducts. Negative liquid secondary ion mass spectrometry (LSIMS) of the nucleotide adducts reflect their molecular weights through formation of intense $(M-H)^-$ ions. Both positive and negative LSIMS gave molecular weight information of the nucleoside adducts. Treatment of the deoxyguanosine 5′-monophosphate and deoxythymidine 5′-monophosphate adducts with alkaline phosphatase produced the corresponding nucleoside adducts as determined by LSIMS. ^{1}H NMR, ^{13}C NMR, and collisionally activated decomposition (CAD) mass spectra were used to determine sites of alkylation. The reactive C-7 position of dehydroretronecine is shown to alkylate N-6 of adenosine, O-2 of deoxythymidine and deoxythymidine 5′-monophosphate, and N-2 of deoxyguanosine 5′-monophosphate. By analogy it is deduced that alkylation occurs at O-2 of uridine and uridine 5′-monophosphate. Cytidine and cytidine 5′-monophosphate do not yield isolable alkylation products under these conditions.

Pyrrolizidine alkaloids are found in a wide variety of plant species of genera that include Senecio, Crotalaria, Heliotropium, Erechtites, Trichodesma, and Amsinckia. These plants are widely distributed throughout the world and have been of particular concern to stockmen because of their hepatotoxic effects in cattle and horses.[1,2] They have also become an emerging public health problem.

Retronecine (I) is the pyrrolizidine nucleus for many pyrrolizidine alkaloids. Most naturally occurring pyrrolizidine alkaloids are mono- or diesters of retronecine. An important structural feature required for toxicity is the presence of a 1,2 double bond. The toxic action of these alkaloids, however, is probably exerted through reactive pyrrole derivatives such as dehydroretronecine (DHR; II).[3]



Senecio jacobaea L. (tansy ragwort) contains the alkaloids jacobine, jacoline, senecionine, seneciphylline, and others.[4] They have been detected in honey produced by bees pollinating tansy ragwort flowers,[5,6] and in milk from cattle[7,8] and goats[9] that ingested ragwort. These alkaloids have been shown to be carcinogenic[10,11] and mutagenic,[12] and their pyrrolic metabolites are known to react with DNA[13] and other cellular constituents.[14,15] A recent paper has shown that dehydroretronecine reacts with guanosine at N-2.[16]

Evidence has shown[3] that the mono- and diesters of DHR are more toxic than DHR itself. The chemistry of alkylation, however, is most often studied with DHR, because alkylation involves the pyrrole nucleus regardless of the leaving groups present. The adducts formed, being polar biological molecules, are invariably nonvolatile and thermally labile. The absence of molecular ions and certain other diagnostic fragment ions in spectra from electron impact mass spectral studies reported by Robertson[16] on DHR-guanosine adducts demonstrates the deficiency of this technique for identification of these compounds. Thus, a "soft" ionization mass spectrometric characterization technique was considered essential. Recently we presented[17] preliminary liquid secondary ion mass spectrometric results on the DHR adducts obtained from deoxyguanosine 5′-monophosphate and adenosine. We now wish to report the results of a complete study on the characterization of covalent DHR-nucleoside and DHR-nucleotide adducts.

Experimental Section

Instrumentation. Positive and negative ion mass spectra were obtained with a Kratos MS-50 high-resolution mass spectrometer with a 23 kG magnet and negative ion switching. The instrument is capable of scanning to m/z 3000 at 8 keV. Typical operating conditions were the following: scan rate 30 s/decade and dynamic resolution $M/\Delta M$ 2500. The spectrometer was interfaced to the LOGOS-II Xerox Sigma 7 data system, permitting real-time assignment of masses.[18]

[⊥] "Covalent adducts" is used wherever appropriate, in order to distinguish a chemical reaction between DHR and nucleosides/nucleotides forming covalent bonds as opposed to "adducts" or "complexes" which are produced under LSIMS conditions. The covention *RS* is used to indicate that the diastereomers (the ribose unit has chiral centers) produced from an S_N1 reaction with the nucleophiles were separated but not distinguished. In cases where the two diastereomers were not separated the convention *RS·SR* is used.

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A primary ion beam produced by a cesium ion gun[19] was used for sputtering of the samples admixed with glycerol. The primary 6-keV cesium ion beam was focused to a spot on the target probe 1–2 mm in diameter.

Samples were dissolved in water, methanol, or dimethyl sulfoxide, and 1 μL of solution was added to about 3 μL of glycerol or glycerol–thioglycerol (1:1) on the probe tip. Excess solvent was pumped away in the vacuum lock of the mass spectrometer. Calibration was achieved with an Ultramark 1621 (PCR, Gainesville, FL). Samples were introduced through the end-plate of the Kratos FD source. The probe tip was made of copper and was cleaned in a phosphoric acid electrolysis bath at 3.5 V.

Positive ion mass spectra were also recorded on a Varian CH-7 single-focusing mass spectrometer equipped with a saddle field Ion Tech., Ltd. fast atom bombardment (FAB) gun using xenon atoms with a mean energy of 7 keV for sputtering. Samples were introduced to the mass spectrometer on a gold plated probe tip through an axially mounted direct insertion lock. The probe tip was cleaned with concentrated nitric acid followed by distilled water. A few micrograms of the sample was directly mixed on the probe tip with glycerol.

CAD spectra were obtained as described[20] from the Midwest Center for Mass Spectrometry, University of Nebraska, with a Kratos MS-50 triple analyzer mass spectrometer. The LSIMS ion source is a standard Kratos design equipped with an Ion Tech atom gun. Samples were dissolved in glycerol and placed on the copper probe tip on the end of a direct insertion probe. The sample was bombarded by 7–8 keV argon or xenon atoms. CAD spectra were obtained with helium gas for collisional activation. CAD spectra were acquired, signal averaged, and output by using software written in the above laboratory for the Kratos DS-55 data system.

Materials and Methods. Retronecine was prepared by hydrolysis of monocrotaline.[21] Dehydroretronecine was synthesized by dehydrogenation of retronecine with chloranil.[22] The crude product was purified by sublimation and recrystallization from hexane–acetone. Nucleotides (free acid form), nucleosides, and alkaline phosphatase (from Escherichia coli, Type III) were obtained from Sigma Chemical Co. Potassium carbonte was reagent grade. Water was double distilled and deionized. Methanol was HPLC grade.

Nucleotide Adducts Preparation. In a typical reaction 0.1 M K_2CO_3 was added to 0.06 mmol of deoxynucleotide in 0.5 mL of water to bring the solution to pH 7.4. After the volume was adjusted to 2.0 mL with water, 0.04 mmol of crystalline dehydroretronecine was added and argon bubbled through the solution for 5 min. The reaction vessel was sealed off from the atmosphere and heated at 60 °C in a water bath for 2 h. If a precipitate formed under these conditions, the solution was centrifuged before preparative HPLC.

The adducts were eluted from a preparative Whatman Partisil M20 10/25 ODS-3 column with 100% water, using a flow rate of 7.2 mL/min. Absorbance was monitored at 220 and 260 nm with a Beckman Model 165 detector. The eluants were lyophilized and frozen until analyses were performed.

Deoxynucleotide adducts were treated with alkaline phosphatase by dissolving 2 mg of adduct in 0.4 mL (pH 9) of Tris buffer (10 mM in $MgCl_2$), adding three units of alkaline phosphatase, and incubating at 37 °C for 1–18 hours. LSIMS spectra were obtained directly on the hydrolysates.

Nucleoside Adducts Preparation. Adenosine and DHR were taken in different proportions in a 0.1 M phosphate buffer (pH 7.4), nitrogen or argon was bubbled into the solution, and the solution was incubated at 37, 60, or 80 °C for several hours. The samples were centrifuged, and products in the supernatant were identified by analytical HPLC on a C_{18} reverse phase (Altex, Ultrasphere, ODS) column with water methanol gradients. Since it appeared from the liquid chromatograms that the ratio of adenosine to DHR or the temperature of reaction had no significant effect on the adducts as long as sufficient reaction time was allowed, in subsequent syntheses 0.05 mmol of DHR, 0.10 mmol of nucleoside, a reaction volume of 5 mL, a temperature of 60 °C, and a time of 2 h were used. The products were isolated by preparative HPLC on a C_{18} reverse phase column (Whatman Partisil M20 10/25 ODS-3) with water–methanol gradients. Fractions containing product peaks were collected. Methanol was removed by rotary evaporation at 40 °C, and products were recovered by lyophilization. Lyophilized samples were frozen until analyses were performed.

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Table I. Significant Peaks Observed in (a) Positive and (b) Negative LSIMS Spectra of Covalent Dehydroretronecine–Nucleoside/Nucleotide Adducts[a]

(a) Positive

nucleoside/ nucleotide adduct	$(M+H)^+$/ $(M_{CH_3}+H)^+$	$(M+Na)^+$/ $(M_{CH_3}+Na)^+$	$(M+K)^+$/ $(M_{CH_3}+K)^+$	$(M-H_2O+H)^+$
guanosine	419/433			
deoxythymidine		400/414	416/430	360
uridine		402	418	362

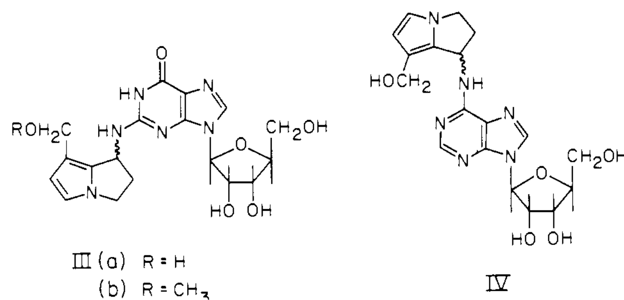
(b) Negative

nucleoside/ nucleotide adduct	$(M-H)^-$/ $(M_{CH_3}-H)^-$	$(M-2H+Na)^-$	$(M-2H+K)^-$
guanosine	417/431		
adenosine	401		
deoxyguanosine 5′-monophosphate	481	503	519
deoxythymidine 5′-monophosphate	456	478	494
deoxyuridine 5′-monophosphate	442	464	480
deoxyadenosine 5′-monophosphate	465		

[a] M = molecular weight of monoalkylated covalent adduct. M_{CH_3} = molecular weight of the methyl ether of monoalkylated covalent adduct.

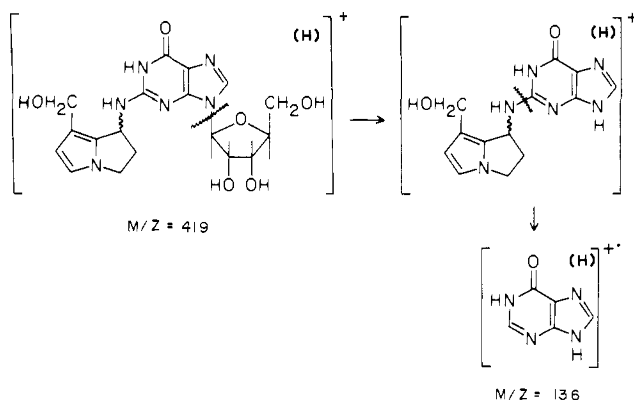
Results

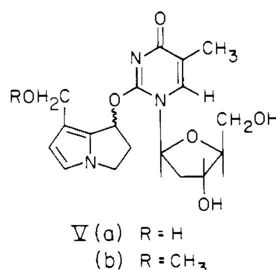
Nucleoside Adducts. Guanosine adducts (IIIa) of DHR were prepared and isolated according to the procedure of Robertson.[16] Liquid chromatographic results of these two adducts were similar to that published. The isolated adducts when analyzed by negative LSIMS yield intense peaks at m/z 417, which is the $(M-H)^-$ ion of the monoalkylated covalent adduct. (LSIMS data are summarized in Table I.) A peak at m/z 431, which presumably is due to (M–H) of the methyl ether derivative (IIIb), was also present in the spectra. This product most likely results from reaction with the methanolic HPLC eluant. Methanol was found to react with DHR to yield small amounts of the C-7 and C-9 dimethyl ethers at 40 °C. The positive ion LSIMS spectrum of this adduct shows an $(M+H)^+$ ion at m/z 419, and this ion when subjected to collisional activation by the MIKES technique[20] produced an abundant ion at m/z 136.[23] The CAD spectrum of the $(M+H)^+$ ion of the C-9 methyl ether (IIIb) also shows an ion at m/z 136. This is most likely due to the fragmentation shown in the scheme below. Such a fragment ion is most reasonable if reaction takes place at the N-2 position of guanosine. This fragmentation and N-2 alkylation were further confirmed by the presence of an ion at m/z 122 in the positive LSIMS spectrum of the covalent adduct formed between guanosine and a synthetic pyrrole without the C-1 hydroxymethyl group.



(23) We thank Dr. M. Gross, University of Nebraska, for these results.

Two product peaks were observed upon HPLC separation of the adenosine–DHR reaction mixture. Their negative LSIMS spectra are identical. The peak at m/z 401 is due to the $(M - H)^-$ ion of the monoalkylated RS adduct (IV). The high-resolution mass spectrum of the permethylated derivative shows peaks at m/z 119 and 149 with exact masses corresponding to fragmentations from adenosine adducts.[24] The proton NMR spectrum of these adducts are identical and show signals at 8.6 and 8.25 ppm for H-2 and H-8, respectively. Relative to the spectrum of adenosine, the H-8 signal of the adducts is shifted downfield by about 0.15 ppm. However, both these resonances are clearly present, and alkylation at any of the purine ring positions is ruled out, N-6 being the only alternative alkylation site. Similar results were obtained with deoxyadenosine adducts.



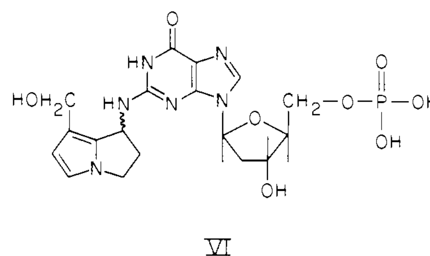


Only one major adduct peak was apparent from the high-pressure liquid chromatogram of the reaction mixture of deoxythymidine and DHR. The positive LSIMS of this adduct shows ions at m/z 400 and 416 and are due to sodiated $(M+Na)^+$ and potassiated $(M+K)^+$ ions of the monoalkylated covalent adducts (Va). Sodium and potassium ion contamination is probably due to the use of a buffer containing potassium dihydrogen phosphate and sodium hydroxide in the synthesis of these adducts. The intense peak at m/z 360 is due to $(M-18+H)^+$, resulting from the loss of a water molecule. Peaks due to loss of a molecule of water from the parent ion are common for nucleosides.[20] The relatively less intense peaks at m/z 414 and 430 are due to the sodiated and potassiated ions, respectively, of the methyl ether derivates (Vb), which presumably result once again from reaction with the methanolic HPLC eluant. A proton NMR spectrum of the adduct shows that the H-6 signal is shifted downfield by 0.11 ppm relative to the H-6 of deoxythymidine. It has been shown[25] that alkylation of uridine at O-2 results in a downfield shift of the H-6 signal by 0.13 ppm. Methylation at N-3 does not affect the H-6 resonance, and ethylation at O-4 results in a significantly greater downfield shift due to extended conjugation of double bonds in the pyrimidine ring. On this basis it is concluded that O-2 alkylation occurs. Further evidence for O-2 alkylation comes from the splitting of the H-1′ triplet at 6.2 ppm of the deoxythymidine moiety into a complex multiplet. This splitting should occur as a result of spacial interactions. Examination of molecular models reveals that spacial interaction is most likely if O-2 alkylation has occurred.

Two uridine adducts—apparently in equal amounts as observed in the liquid chromatogram—were isolated from the reaction of uridine and DHR. Their LSIMS spectra were identical. These adducts show peaks at m/z 362, 402, and 418 in their positive LSIMS spectra. As in the case of deoxythymidine adducts, these peaks correspond to $(M-18+H)^+$, $(M+Na)^+$, and $(M+K^+)$ ions of the monoalkylated products.

Nucleotide Adducts. Deoxyguanosine 5′-monophosphate reacted with DHR to yield one major product peak in the liquid chromatogram. Negative LSIMS of this product shows ions at m/z 481, 503, and 519 due to $(M-H)^-$, $(M-2H+Na)^-$, and $(M-2H+K)^-$ ions, respectively, of the monoalkylated adducts. Alkaline phosphatase treatment of the product produced a monoalkylated deoxyguanosine–DHR adduct as observed by LSIMS. This means alkylation did not take place on the phosphate group.

In an 80-MHz spectrum of the above adduct in D_2O, H-7 of the DHR residue appears as a doublet of doublets and is shifted downfield by 0.25 ppm with respect to that of the unreacted material. In a 360-MHz NMR spectrum, the H-7 proton of the adduct appears at 5.38 ppm, as a sextuplet. Decoupling of one of the H-6 protons at 2.3 ppm converts this multiplet to a doublet of doublets. Decoupling of the other H-6 proton at 2.8 ppm converts it to a three-line signal. The latter signal could result from overlapping of another set of doublet of doublets. These observations would then be explained on the basis that the product corresponding to the one adduct peak in the liquid chromatogram is in fact the *RS·SR* mixture, resulting from reaction of the nucleophile at the C-7 position of DHR via an S_N1 reaction. A ^{13}C NMR spectrum of deoxyguanosine 5′-monophosphate shows a signal for C-6, i.e., the carbonyl carbon, at 159.5 ppm. In the spectrum of the adduct the resonance line for the carbonyl carbon appears at 161.5 ppm, thus ruling out alkylaton at O-6 to form an aromatic ether. The positive ion LSIMS spectrum of the adduct produces an $(M+H)^+$ ion at m/z 483. As in the case of guanosine, a CAD spectrum of the $(M+H)^+$ ion at m/z 483, produced by LSIMS, showed an ion at m/z 136. It is concluded that N-2 (VI) alkylation has taken place.



VI

One product as observed in the liquid chromatogram was isolated from the reaction of deoxythymidine 5′-monophosphate with DHR. This corresponded to a monoalkylated adduct characterized by a very intense $(M-H)^-$ ion at m/z 456 in the negative LSIMS spectrum. Less intense potassiated $(M-2H+K)^-$ and sodiated $(M-2H+Na)^-$ ions at m/z 494 and 478, respectively, were observed. A peak observed at m/z 586 corresponds to the glycerol adduct ion $(M-2H+K+92)^-$. Alkaline phosphatase treatment of the monoalkylated covalent adduct produces the corresponding covalent nucleoside adduct as observed by LSIMS of the hydrolysate, thus ruling out alkylation at the phosphate group. NMR data are very similar to those obtained with the deoxythymidine adducts, and arguments in favor of O-2 alkylation hold true here too.

Deoxyuridine 5′-monophosphate reacted with DHR to give one product peak in the liquid chromatogram. An ion at m/z 442 in the negative ion LSIMS spectrum due to $(M-H)^-$ and sodiated $(M-2H+Na)^-$ and potassiated $(M-2H+K)^-$ ions at m/z 464 and 480, respectively, suggest it is a monoalkylated adduct. Relatively less intense glycerol adduct ions of the above ions were also observed at m/z 534, 556, and 572. Finally, deoxyadenosine 5′-monophosphate reacted with DHR to yield one adduct as observed

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from the liquid chromatogram and an intense ion at m/z 465 in the negative ion LSIMS corresponding to $(M-H)^-$ of the monoalkylated covalent adduct.

Discussion

Ultimate chemical carcinogens are known to involve mechanisms that are predominantly S_N1 in character in their reactions with cellular macromolecules—the latter being the nucleophiles.[26] Preliminary experiments from our laboratory[27] and subsequent experiments have shown that the C-7 position of DHR is the primary site of attack by nucleophiles. All alkylation reactions of nucleosides and nucleotides by DHR carried out in this laboratory proceed via an attack on the C-7 carbonium ion. A nucleophilic attack on the C-7 carbonium ion should produce the *RS*·*SR* mixture of adducts. NMR data in the case of guanosine 5′-monophosphate and the results of studies with guanosine[16] show that indeed *RS*·*SR* product mixtures were obtained. Other pyrroles produced upon biotransformation of pyrrolizidine alkaloids have also been reported[22] to react at C-7, yielding enantiomeric products. Rat liver microsomal transformation of jacobine and lasiocarpine—the pyrrolizidine alkaloids derived from the diastereoisomers retronecine and heliotridine, respectively—produced a racemic mixture of dehydroretronecine and dehydroheliotridine.[28]

Adenosine reacts at the N-6 position, and two products of identical mass and NMR spectra were obtained. They are, therefore, the *RS* and *SR* products from the reaction at the C-7 position of DHR. Uridine also produced two products of identical molecular weight. In the case of deoxythymidine and deoxythymidine 5′-monophosphate we showed that reaction takes place at O-2. Hence, considering the structural similarity between uridine and deoxythymidine it is concluded that *RS* and *SR* products are formed due to reaction at C-7 of DHR with O-2 of uridine.

Cytidine and cytidine 5′-monophosphate did not yield any isolable products. By an indirect test procedure Mattocks and Bird[29] have found reaction to take place between DHR and cytidine at pH 5. However, the products were not characterized. Since reaction at pH 7.4 was not observed in these studies, and since some reaction has been observed at pH 5, solution pH may be critical for reaction to occur.

Characterization of reaction products of pyrrolic metabolites with cellular target molecules contributes to the understanding of the mechanism of their toxic effects. Cross-linking between reactive pyrroles and DNA has been implicated.[13,32] To the best of our knowledge, however, cross-linked or bialkylated products have not been identified. As found in this study, dehydroretronecine; although having bifuncational alkylating capacity, apparently yields only monoalkylated products from its interaction with common nucleosides and nucleotides. At neutral pHs the binding sites on the bases of nucleosides apparently are the same as those on the corresponding nucleotides. Not all alkylating agents behave in this manner, however: The structurally similar reactive metabolite of mitomycin C does not alkylate uridine under acidic conditions whereas uridine 5′-monophosphate is alkylated at the phosphate group.[30] Other differences in the reactions of reductively activated mitomycin C from those of oxidatively activated pyrrolizidine alkaloids should also be noted. The products after enzymatic digestion of calf thymus DNA modified by mitomycin C under neutral reaction conditions include an adensonine adduct substituted at N-6 and two guanosine adducts substituted at N-2 and O-6.[31] Reaction of deoxyguanosine 5′-monophosphate with activated mitomycin C under neutral conditions yields the O-6 adduct.[33] These differences may be accounted for by minor differences in the activation energies of the electrophiles.

This study demonstrates the value of LSIMS in the analyses of trace quantities of biologically important compounds. Early mass spectrometric studies involving covalent adducts between alkylating agents and the bases from DNA have often required elaborate derivatization schemes. As noted previously,[16] these were not always successful. The use of LSIMS would be a wise choice for these types of investigations, particularly when relatively high molecular weight covalent adducts are involved.

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Registry No. IIIa (isomer 1), 94781-93-8; IIIa (isomer 2), 94781-94-9; IIIb (isomer 1), 94781-95-0; IIIb (isomer 2), 94781-96-1; IV (isomer 1), 94781-97-2; IV (isomer 2), 94781-98-3; Va (isomer 1), 94781-99-4; Va (isomer 2), 94782-00-0; Vb (isomer 1), 94782-01-1; VI, 94782-02-2; Vi (isomer 1), 94800-13-2; VI (isomer 2), 94782-03-3; dehydroretronecine, 23107-12-2; guanosine, 118-00-3; adenosine, 58-61-7; deoxythymidine, 50-89-5; deoxyguanosine 5′-monophosphate, 902-04-5; uridine, 58-96-8; deoxythymidine 5′-monophosphate, 365-07-1; deoxyuridine 5′-monophosphate, 964-26-1; deoxyadenosine 5′-monophosphate, 653-63-4.

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