Mass Spectrometry of Nucleic Acid Components. Analogs of Adenosine

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Abstract: The mass spectra of adenosine and 32 of its analogs have been studied in detail. Principal fragmentation pathways for structurally significant ions have been determined and decomposition mechanisms postulated, based on metastable transitions, deuterium and substituent labels, and high-resolution mass spectra. The major ions M − 30, base + 44, and base + 30 are proposed to arise from initial transfer of sugar hydroxyl hydrogens to the charge-localized purine base. Methylation at N^6 is characterized by elimination of CH_3N^6 with rearrangement of either hydrogen or a methyl group as previously reported for the corresponding bases. 2′-O-Methylation leads to a unique sugar fragment resulting from elimination of the base plus a 3′- or 5′-hydroxyl hydrogen. Anomers are readily distinguished by their mass spectra, but steric orientation of sugar hydroxyls cannot be determined directly. However the abundance of the M − 30 ion was found to depend strongly on the steric accessibility of C-5′ to the base.

The characterization or structure determination of nucleosides and their analogs plays an important role in several areas of chemistry and biology, notably in the synthesis of nucleoside analogs for studies of their tumor inhibitory properties and in the determination of structure and sequence of modified nucleosides in ribonucleic acid (RNA). Much current interest is being shown in the structure and biological function of transfer RNA, which contains a high proportion of modified nucleosides in addition to the four normally found. In these cases reliance is most frequently placed on ultraviolet spectra and chromatographic characteristics for characterization. However, if a new compound is encountered, often in very small quantity, additional time- and sample-consuming methods must usually be employed.

For these reasons the sensitivity, speed, and objectivity of mass spectrometry offer considerable potential for dealing with structural problems of nucleic acid components. Development of the mass spectrometry of natural products has in the past been largely within the domain of chemistry rather than biology. Therefore considerable attention has been devoted to the mass spectrometry of certain classes of compounds, such as steroids[1] and fatty acid esters,[2] while systematic investigation of the basic fragmentation behavior of nucleosides or other nucleic acid components have been limited to several studies of limited scope.[3-6] Detailed reports on the mass spectra of purine and pyrimidine bases have appeared,[7] as well as two communications on trimethylsilyl derivatives of mono-[8] and dinucleotides.[9]

The potential value of mass spectrometry in this field[10] may be judged by the considerable usage of the technique in structural problems,[7d,11-19] often playing a central role,[20-27] based almost solely on the first communication which described structure correlations

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of the principal fragment ions from several nucleosides, including adenosine (**1**) and 2′-deoxyadenosine (**2**).[3] With several exceptions[3,4,6,15,19,21,25] few detailed proposals regarding mechanisms of ion formation in free nucleosides have been made. Unique aspects of the interpretation of nucleoside mass spectra through high-resolution element maps have been discussed by Biemann and Fennessey[28] and Tsunakawa.[29]

The present study was undertaken to systematically assess the structures and mechanisms of formation of principal fragment ions in nucleoside mass spectra, as well as ions closely associated with structural modification, notably methylation.

The compounds studied were generally limited to structural variants of a single base, adenine, for two reasons. First, by keeping the nature of the base constant, competitive effects from other structural features can be more easily distinguished. Although the nature of the base (*e.g.*, purine *vs.* pyrimidine) may govern the abundances of some ions, the same basic fragmentation pathways appear to exist for all nucleosides. Second, adenosine analogs appear to comprise a substantial proportion of modified nucleosides which have been identified in transfer RNA. It should further be noted that the more polar nucleosides such as cytidine and guanosine are not directly amenable to mass spectrometry with presently available sample introduction techniques due to their high polarity and hence low volatility.

As an alternative, conversion of nucleosides to trimethylsilyl derivatives provides compounds which are sufficiently volatile[30] for introduction to the mass spectrometer through a gas–liquid chromatograph, thus also reducing requirements for sample purity, and which yield structurally informative mass spectra.[8] Although derivatization appears to be the most generally satisfactory approach,[31] many instances may occur in which the uncertainties of further sample handling and chemical modification are inappropriate, in which case direct vaporization of the free nucleoside is desirable if volatility permits.

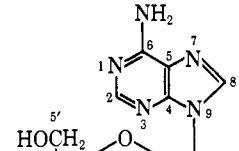
Preparation of Deuterium-Labeled Nucleosides. Isotopic labeling plays a well-established, key role in the interpretation of mass spectra, both in the case of model compounds and structural unknowns.[32] The identification of carbon atoms in a given fragment ion can often be established by the use of substituent (*i.e.*, methyl) labels or through the mass spectra of thio or amino analogs, as demonstrated in the following section. However, a detailed consideration of fragmentation mechanisms and ion structures more frequently requires the use of deuterium-labeled compounds. While the systematic incorporation of deuterium into a nucleoside skeleton represents a formidable synthetic problem, the exchange of labile hydrogens by solution of the compound in D_2O is experimentally simple[3] in most cases. This method, which is particularly applicable to nucleosides and other compounds which possess several active hydrogen atoms, is one of the most potentially useful but seldom used labeling techniques for mass spectrometry. The principal experimental difficulty arises from partial reexchange of deuterium in the ion source after sample vaporization. This problem can be minimized if the path between the sample holder and electron beam is short and unobstructed, and if the walls of the ion source are sufficiently cool (with respect to the vaporization temperature of the sample) to preclude extensive collisions between sample molecules and the wall.

Table I. Exchange of Labile Hydrogen by Deuterium in Nucleosides

Compound	No. exchangeable hydrogens	% total D incorporation; mol %, d_0, d_1, d_2, d_3, etc.
N^6,N^6-Dimethyladenosine[a]	4	[a]36.4%; 11.5, 27.7, 25.7, 9.0, 0.6
		[b]62.7%; 2.5, 12.2, 30.9, 36.1, 17.1
2′-O-Methyladenosine[b]	5	[b]77.8%; 0.0, 1.2, 5.8, 19.9, 39.3, 31.9
5′-Deoxyadenosine[b]	5	[b]59.0%; 1.5, 7.8, 19.2, 37.7, 27.6, 5.1
2′,5′-Dideoxyadenosine[b]	4	[b]56.7%; 4.8, 15.3, 34.6, 34.7, 9.5, 1.1

[a] Exchange by CH_3OD in ion source, 210–240°. [b] Exchange by solution in D_2O at 60°; also CH_3OD treatment in ion source.

For a given incorporation level of deuterium, the ratio of M − 1 (from incomplete exchange) to M increases as the number of labile hydrogens in the molecule increases, assuming nearly equal probability of exchange for each such hydrogen. For a molecule having a relatively large number of exchangeable hydrogens (*e.g.*, adenosine which has 6, including H-8), the presence of several prominent isotope peaks due to incompletely labeled species may cause difficulty in obtaining quantitative values of deuterium content for some fragment ions. For these reasons we have attempted to obtain maximum final deuterium incorporation into several nucleosides, which then serve as models for the interpretations made in the following section. Table I shows labeling results from several representative compounds. N^6,N^6-Dimethyladenosine (**3**) was



1, R = OH
2, R = H

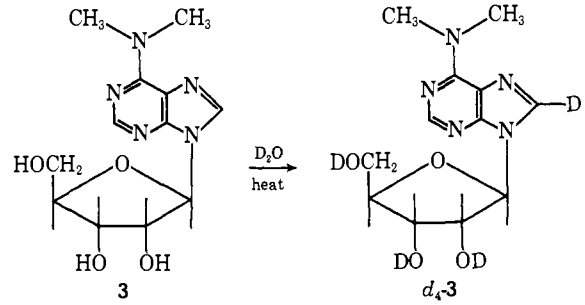


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Table II. Selected Ions from the Mass Spectra of Adenosine Analogs

Compound[a]	m/e/relative intensity M	c	d	h	f	b + H	b + 2H	%Σ40/%RI
Adenosine (1)	267/3.3	237/6.5	178/35.1	164/69.9	148/23.0	135/100	136/77.2	0.12
2′-Deoxy-β-adenosine (2)	251/3.8	221/6.1	162/37.0	164/12.0	148/1.3	135/100	136/30.0	0.29
N^6,N^6-Dimethyladenosine (3)	295/20.4	265/3.7	206/27.3	192/56.8	176/7.2	163/53.9	164/49.1	0.18
2′-C-Methyladenosine (4)	281/1.6	251/2.3	192/13.9	164/100	148/9.1	135/86.0	136/48.4	0.17
3′-C-Methyladenosine (5)	281/1.8	251/14.8	178/48.1	164/62.7	148/11.6	135/100	136/72.8	0.17
5′,5′-Di-C-methyladenosine (6)	295/0.3	237/7.8	178/20.3	164/16.2	148/0.9	135/100	136/23.3	0.29
9-β-Ribofuranosylpurine (7)	252/0.8	222/3.8	163/25.1	149/85.7	133/11.4	120/32.1	121/100	0.18
1-Methyladenosine (8)	281/7.6	251/0.8	192/5.2	178/4.6	162/1.9	149/100	150/17.9	0.30
N^6-Methyladenosine (9)	281/7.7	251/4.8	192/30.4	178/68.0	162/10.1	149/100	150/73.3	0.13
8-Methylaminoadenosine (10)	296/13.0	266/0.5	207/3.6	193/4.8	177/2.8	164/100	165/17.0	0.35
N^6-(3-Methyl-2-butenyl)adenosine (11)	335/51.0	305/1.9	246/17.0	232/24.0	216/0.6	203/67.0	204/17.0	0.10
3′-Thioadenosine (12)	283/13.0	253/0.9	178/2.5	164/35.4[b]	148/7.5	135/100	136/83.0	0.16
4′-Thioadenosine (13)	283/0.1	253/71.5	178/23.0	180/96.0	148/19.0	135/54.0	136.100	0.12
2′-O-Methyladenosine (14)	281/6.2	251/25.8	192/74.5	164/46.5	148/15.2	135/100	136/89.2	0.11
3′-O-Methyladenosine (15)	281/6.2	251/13.0	178/30.0	164/95.0	148/11.0	135/100	136/98.0	0.13
3′-Deoxyadenosine (16)	251/3.8	221/7.9	178/14.8	164/57.4	148/7.3	135/100	136/43.7	0.20
5′-Deoxyadenosine (17)	251/9.0	221/0.0	178/18.0	164/96.0	148/6.8	135/100	136/96.0	0.15
2,′3′-Dideoxyadenosine (18)	235/5.1	205/7.2	162/23.0	164/3.4	148/3.5	135/100	136/59.0	0.21
2′,5′-Dideoxyadenosine (19)	235/6.5	205/0.0	162/13.0	164/4.6	148/0.8	135/100	136/35.0	0.26
9-(5′-Deoxy-α-xylofuranosyl)adenine (20)	251/5.9	221/0.0	178/77.0	164/75.0	148/7.6	135/100	136/54.0	0.15
9-α-Xylofuranosyladenine (21)	267/4.3	237/0.0	178/42.0	164/38.0	148/9.1	135/100	136/45.0	0.23
2′-Deoxy-α-adenosine (22)	251/4.4	221/1.6	162/36.0	164/6.1	148/1.1	135/100	136/30.0	0.29
9-β-Xylofuranosyladenine (23)	267/5.1	237/2.8	178/38.0	164/66.1	148/12.8	135/100	136/93.8	0.17
9-(5′-Deoxy-β-allofuranosyl)adenine (24)	281/4.6	237/1.4	178/20.1	164/68.5	148/6.7	135/100	136/77.0	0.18
9-β-Psicofuranosyladenine (25)	297/0.8	267/2.2	208/1.9	194/8.7	178/0.5	135/100	136/45.4	0.21
N^6,N^6-Dimethyl-3′-amino-3′-deoxyadenosine (26)	294/9.4	264/0.9	206/17.0	192/53.0	176/1.6	163/30.0	164/100	0.19
N^6,N^6-Dimethyl-2′-O-methyladenosine (27)	309/25.0	279/9.4	220/54.0	192/40.0	176/3.4[b]	163/60.0	164/51.0	0.12
N^6-Methyl-3′-deoxyadenosine (28)	265/7.2	235/5.4	192/14.2	178/44.5	162/6.3	149/100	150/42.0	0.19
2′-O-Methyl-5′-deoxyadenosine (29)	265/14.5	235/2.2	192/15.8	164/80.0	148/11.0	135/80.0	136/100	0.07
2′-O-Methyl-3′-deoxyadenosine (30)	265/4.7	285/18.0	192/24.2	164/52.5	148/6.5	135/100	136/67.0	0.11
9-β-Arabinofuranosyladenine (31)	267/1.1	237/1.1	178/8.9	164/40.0	148/5.0	135/100	136/27.6	0.19
9-β-Allopyranosyladenine (32)	297/4.9	267/0.8	178/6.4	164/89.0	148/6.5	135/100	136/20.0	0.19
9-β-Galactopyranosyladenine (33)	297/5.7	267/0.5	178/9.4	164/81.0	148/8.1	135/100	136/71.0	0.18

[a] All compounds of D configuration. [b] Doublet; relative intensity corrected.

chosen as the primary model compound since it has only one exchangeable hydrogen in the base, where the presence of a label is less important for most interpretations.

The highest incorporation was generally obtained by first prelabeling the compound by solution in warm D_2O, which was removed under vacuum and heat. Then to minimize deuterium loss during sample introduction, CH_3OD was simultaneously admitted to the ion source through a reservoir inlet. Although any isotope distribution which contains substantial amounts of lower labeled species may overlap adjacent nominal masses, these labeling techniques are sufficient to unambiguously establish the number of active hydrogens in a molecule of unknown structure, and to ascertain the principal mass shifts of most major ions.

Discussion of Mass Spectra

The interpretations presented utilize data obtained from deuterium, oxygen-18, and substituent labeling, and exact mass measurement of the entire spectra of most compounds. Mechanistic proposals are based in most cases on the concept of charge localization[33] as a primary factor in fragmentation processes.

The mass spectra of all compounds examined exhibited molecular ions (M), in a wide range of abundances (~0.3–51.0% relative intensity), as shown in Table II. Not surprisingly the lower limits of M abundances are found in compounds **4**,[34] **5**,[35] and **6**[36] which contain methyl branching in the ribose skeleton.[37] On the other hand, M is generally enhanced by the inductive stabilization provided by nitrogen and alkyl substitution in the base, as shown by comparison of **7** with **1**, **3**, and **9–11** in Table II. The presence of sulfur has a marked effect on molecular ion abundances, depending on the position of thiation. As is common for sulfur-containing compounds[38] the molecular ion of 3′-thioadenosine (**12**)[39] is more intense than its oxygen analog adenosine (**1**). However in 4′-thioadenosine (**13**)[40] M is essentially absent because of preferred stabilization of a fragment ion (c, discussed in the following section) by the 4′-heteroatom. Correct identification of the molecular ion will usually permit determination of its elemental

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(37) We are grateful to Dr. F. W. Holly for generous gifts of compounds **4–6**.

(38) Reference 33a, Chapter 7.

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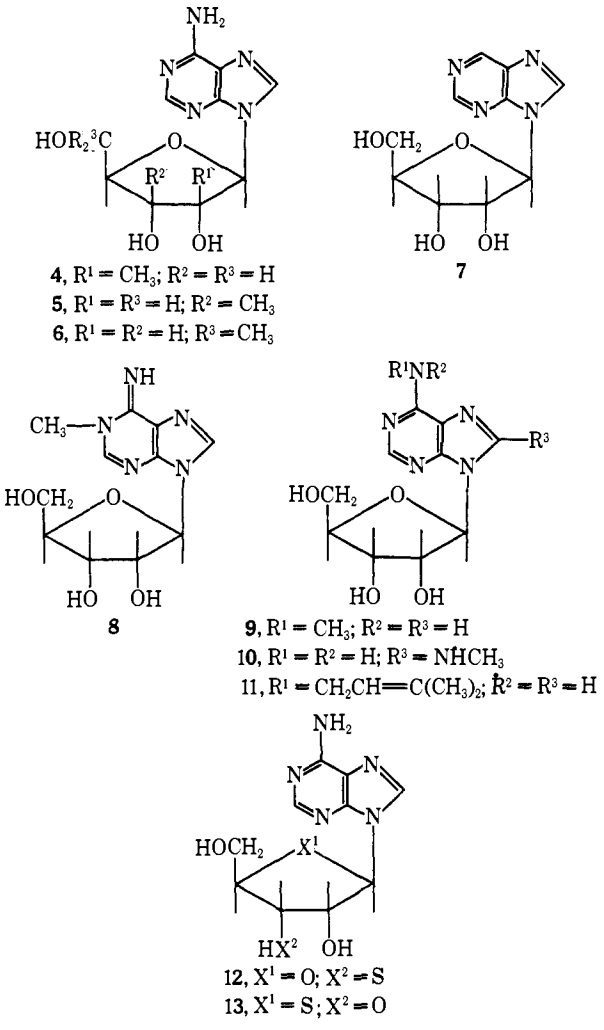
(40) E. J. Reist, D. E. Gueffroy, and L. Goodman, *ibid.*, 86, 5658 (1964).



composition by measurement of its exact mass. For instance, for adenosine there are four possible elemental compositions within 10 ppm of the molecular ion, *m/e* 267.0968: $C_{13}H_{11}N_6O$, $C_{12}H_{15}N_2O_5$, $C_9H_{17}NO_8$, and $C_{10}H_{13}N_5O_4$. Of these, the last is chosen as the only possibility for a purine nucleoside if compositions containing fewer than four nitrogens and three oxygens are excluded as unlikely. With establishment of the correct molecular empirical composition, similar considerations are facilitated for composition assignments for the remainder of the high-resolution spectrum.[28]

Principal fragmentation routes from the molecular ion involve bond breakage in the sugar moiety, reflecting resistance of the aromatic nucleus toward decomposition.[3] The most structurally significant ions therefore consist of the purine base (b) plus various portions of the sugar skeleton. Identification of the sugar skeleton carbons and heteroatoms in this series constitutes a powerful means of specifically locating substituents or isotopic labels[23] in the sugar. With the exception of 2′-O-methyl compounds, the sugar fragment itself (ion s) plays a minor role in the fragmentation of purine nucleosides, although in pyrimidine nucleosides its presence is more evident.[3] In the present case, the sugar fragment (s, *m/e* 133 in ribosides) is of low abundance but is readily detected as *m/e* 133.0501 ($C_5H_9O_4$) in high-resolution spectra.

Loss of a hydroxyl radical from M to yield the minor ion a is commonly observed in most spectra, such as that of adenosine (Figure 1), which corresponds closely

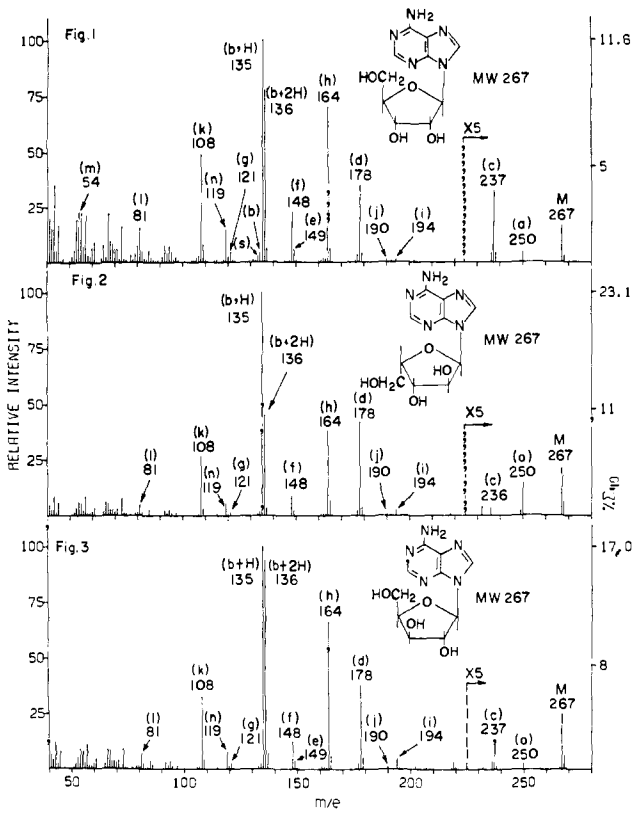
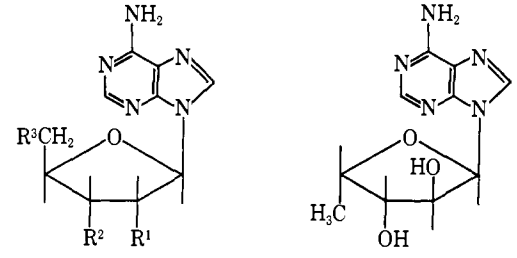


Figure 1. Mass spectrum of adenosine (**1**).
Figure 2. Mass spectrum of 9-α-D-xylofuranosyladenine (**21**).
Figure 3. Mass spectrum of 9-β-D-xylofuranosyladenine (**23**).

to that obtained on a time-of-flight instrument.[3] A specific hydroxyl group is probably not involved in any one molecule, as evidenced by the occurrence of ion a in various cases in which one of the three sugar hydroxyls is not available: 2′- and 3′-O-methyladenosine (**14** and **15**)[41] and 3′- and 5′-deoxyadenosine (**16**[42] and **17**[43]). The spectrum of **1** labeled with 26 mol % oxygen-18 in position 5′[44] shows C-5′ O cleavage



to be approximately statistical (*i.e.*, 1 in 3) since 17 mol % O^{18} is retained in ion a. This is not in accordance with ion stability considerations, which would

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(44) We are indebted to Dr. R. Caprioli of Professor D. Rittenberg's laboratory for a sample of this compound.

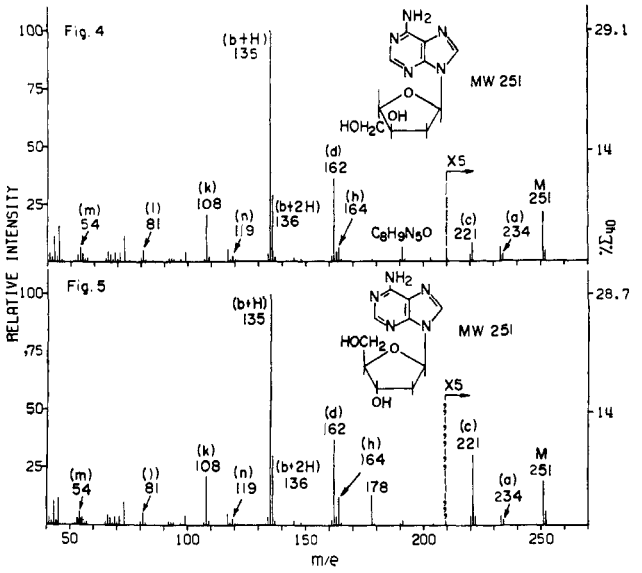
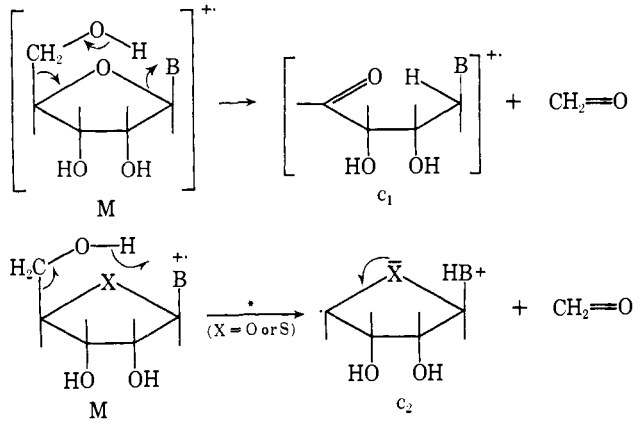


Figure 4. Mass spectrum of 2′-deoxy-α-adenosine (**22**).
Figure 5. Mass spectrum of 2′-deoxy-β-adeosine (**2**).

favor secondary (C-2′ or 3′) over primary carbonium ions (C-5′), indicating that additional factors may be involved, such as stabilization of a 5′ ion by N-3.

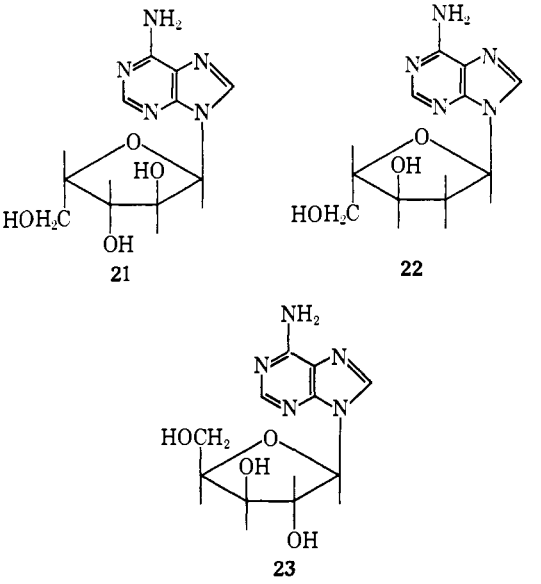
A small peak 18 mass units below the molecular ion due to loss of H_2O was observed in many cases. Although its relative intensity remains generally constant with time, indicating it to be a fragment ion, thermal dehydration cannot be precluded because of the high temperatures of vaporization required for most compounds. One of the most volatile nucleosides studied, 2′,3′-dideoxyadenosine (**18**)[45] exhibits one of the largest M − 18 peaks (6.3% relative intensity, 1.5% Σ).

Elimination of the elements of formaldehyde (30 mass units) from the 5′-hydroxyl group provides ion c, which is an important structural indicator of the 5′ position. Involvement of the 5′ group[3] is verified by the absence of c in the spectra of the three 5′-deoxy compounds **17**, **19**, and **20**, and the absence of O^{18} in c in the spectrum of 5′-O^{18}-**1**. Deuterium labeling revealed the retention of all labile hydrogens in the molecule thus requiring retention of the 5′-O-hydrogen. Several mechanisms can be formulated, the following of which (M → c_1) was presented in 1962[3], without consideration of charge localization or other now common mechanistic nomenclature.



A second alternative is possible (M → c_2), based on the plausible assumption that the charge in the molecular ion is largely concentrated in the aromatic purine nucleus. The feasibility of c_2 as opposed to c_1 is further enhanced by the steric proximity of the base to the 5′-O-hydrogen in the *syn* form[46] of the molecule. Examination of molecular models constructed from Corey–Pauling–Koltun (CPK) space-filling atomic models [49] shows this distance to be approximately <1 Å, within suitable range for hydrogen transfer. On the other hand, the distance between the 5′-O-hydrogen and C-1′ is >3 Å, too great for M → c_1 to proceed without the necessity of postulating opening of the ribose ring for greater conformational flexibility.

The mechanistic model M → c_2 not only predicts that the occurrence of c requires a labile hydrogen in the 5′ position, but also that it be sterically accessible to the base. To test this latter requirement the mass spectra were determined of two nucleosides in which the orientation of the 5′-hydroxyl group was "*trans*" to the base across the furanose ring: 9α-xylofuranosyladenine (**21**)[50] and 2′-deoxy-9α-adenosine (**22**).[51] Ion c is absent in the spectrum of **21**, Figure 2, while in the spectrum of the β anomer (**23**), Figure 3, its abundance



is 2.8%, 0.5% Σ (Table II). In the case of the deoxyanomer **22** ion c is present (confirmed by measurement of exact mass), though as shown in Figure 4 its abundance is reduced approximately fourfold in comparison with 2′-deoxyadenosine (**2**), Figure 5. From these data we conclude that the mechanism M → c_2 predominates over other routes which do not require spatial proximity of the base to the 5′ substituent.

Methyl branching at C-5′ in 5′,5′-di-C-methyladenosine (**6**) results in a significant peak at M − CH_3 (2.7% relative intensity, 0.7% Σ), *m/e* 280, but does not prevent the formation of ion c. Likewise, additional

(45) G. L. Tong, W. W. Lee, and L. Goodman, *J. Org. Chem.*, **30**, 2854 (1965).

(46) Both *syn* and *anti* conformations of the molecule[47] must be assumed to exist during fragmentation because of the high energy content of the ions, and the apparently low energy barriers to rotation about the glycosidic bond.[48]

(47) J. Donohue and K. N. Trueblood, *J. Mol. Biol.*, **2**, 363 (1960).

(48) A. E. V. Haschemeyer and A. Rich, *ibid.*, **27**, 396 (1967).

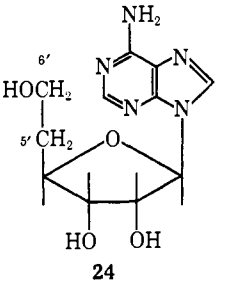
(49) W. L. Koltun, *Biopolymers*, **3**, 665 (1965).

(50) We thank Dr. Leon Goodman for generous gifts of **21** and **22**.

(51) R. H. Iwamoto, E. M. Acton, and L. Goodman, *J. Org. Chem.*, **27**, 3949 (1962).

presence of another methylene group in C-5′ in (9-(5′-deoxy-β-D-allofuranosyl)adenine (homoadenosine),[52] **24**) has no influence on the loss of C-5′ and 6′ as 44 mass units to produce c, *m/e* 237. Stability of the radical site at C-4′ in c_2 may therefore be considered to play a role in the formation of c since elimination of formaldehyde from the molecular ion of **24** does not occur to any significant extent, the resulting 5′ radical not being stabilized by the C-4′ heteroatom. The proposed structure c_2 is further corroborated by the high abundance of ion c in 4′-thioadenosine (**13**, see Table II), which can be attributed to the greater stabilizing influence of sulfur compared with oxygen.[53]

Loss of the C-5′ moiety (M − CH_2OH) by simple cleavage to give a secondary ion stabilized by the ribose ring oxygen frequently occurs as a very minor but general process. In the mass spectrum of the antibiotic psicofuranine, **25**, the abundance of the M − 31 ion is increased over adenosine (**1**) (0.9 *vs.* 0.1% Σ), presumably because loss of the 1′-hydroxymethylene radical (as opposed to 5′) provides better charge stabilization through participation of N-9 of the adenine moiety.



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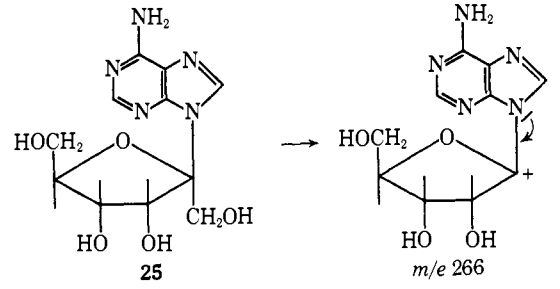


25 *m/e* 266

Of major structural significance is the ion which occurs at a mass value equal to the mass of the base (b) plus 44 mass units in the mass spectra of ribosides[3] (ion d; *m/e* 178 in Figure 1). Since d contains carbon atoms 1′ and 2′ plus a rearranged hydroxyl hydrogen, its mass shifts downward 16 units in the spectra of 2′-deoxypentosides (*m/e* 162, Figure 5) and upward 14 mass units in the case of 2′-O-methyl nucleosides (*m/e* 192, Figure 10). Earlier data obtained from a time-of-flight mass spectrometer (from which metastable ion transitions are not recorded) led to the postulate that the loss of 89 mass units in formation of ion d mechanistically followed the production of c by simple cleavage of the C-2′,3′ bond (see equation 1). Any attempt to derive a general mechanism for the formation of ion d must be based on the following considerations and experimental observations. (1) M → d_1 does not represent an entirely satisfactory mechanism, because (i) the 5′-hydroxyl hydrogen cannot be transferred to C-1′ without opening of the furanose ring and (ii) stabilization of the charge in d_1 would depend strongly on the unshared electrons of the

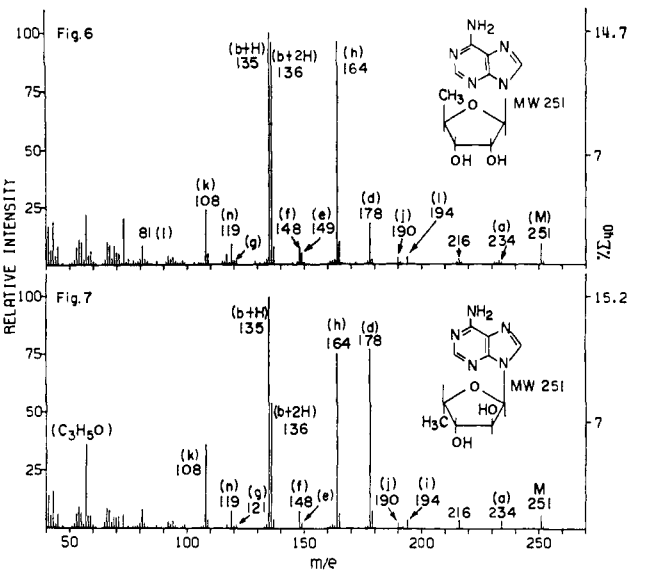
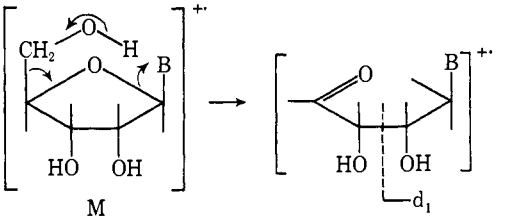


Figure 6. Mass spectrum of 5′-deoxyadenosine (**17**).
Figure 7. Mass spectrum of 9-(5′-deoxy-α-furanosyl)adenine (**20**).



M (1)

hydroxyl oxygen at C-2′, while in fact the abundance of d in 2′-deoxyadenosine (Figure 5) is greater than in the case of adenosine, Figure 1. (2) Ion d is prominent in the mass spectra of several compounds which contain no 5′-hydroxyl group: 5′-deoxyadenosine (**17**), Figure 6; 9-(5′-deoxy-D-xylofuranosyl)adenine (**20**), Figure 7, in which the C-5′-base distance is maximized; and 2′,5′-dideoxyadenosine (**19**). In each of the above cases deuterium-labeling experiments reveal that to a large extent a labile hydroxyl hydrogen is transferred in the formation of ion d. (3) As shown in Table II, ion d is likewise formed in the spectra of compounds which are unable to provide an active hydrogen from position 3′: 3′-deoxyadenosine (cordycepin) (**16**), 3′-O-methyladenosine (**15**), and 2′,3′-dideoxyadenosine (**18**). (4) The generation of d directly from ion c was indicated by weak metastable transitions only in the case of 3′-deoxyadenosine (**16**) (m^* = $178^2/221$ = 143.4; 143.5, fd) and N^6-methyl-3′-deoxyadenosine (**28**) (m^* = $192^2/235$ = 156.9; 157.0, fd). (5) As a general fragmentation pathway ion d further decomposes by (i) loss of CHO to yield ion e which has the composition base + CH_3 and (ii) expulsion of CH_2CO to ion b + 2H. Metastable transitions for d → e were observed in the mass spectra of numerous compounds (*e.g.*, **1**, **9**, and **24**), also for d → b + 2 (*e.g.*, in **1**, **5**, **8**, and **25**).

It is evident from these data that more than a single mechanism exists for the general transition M → d,

(52) K. J. Ryan, H. Arzoumanian, E. M. Acton, and L. Goodman, *J. Amer. Chem. Soc.*, 86, 2503 (1964).
(53) K. Biemann, "Mass Spectrometry," McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p 87.

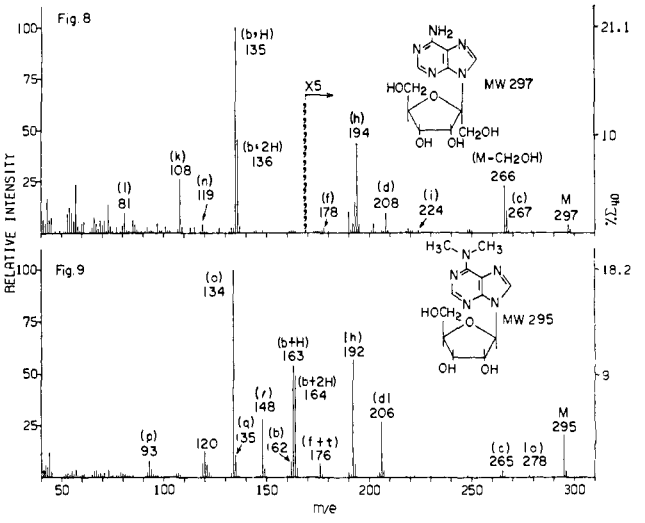
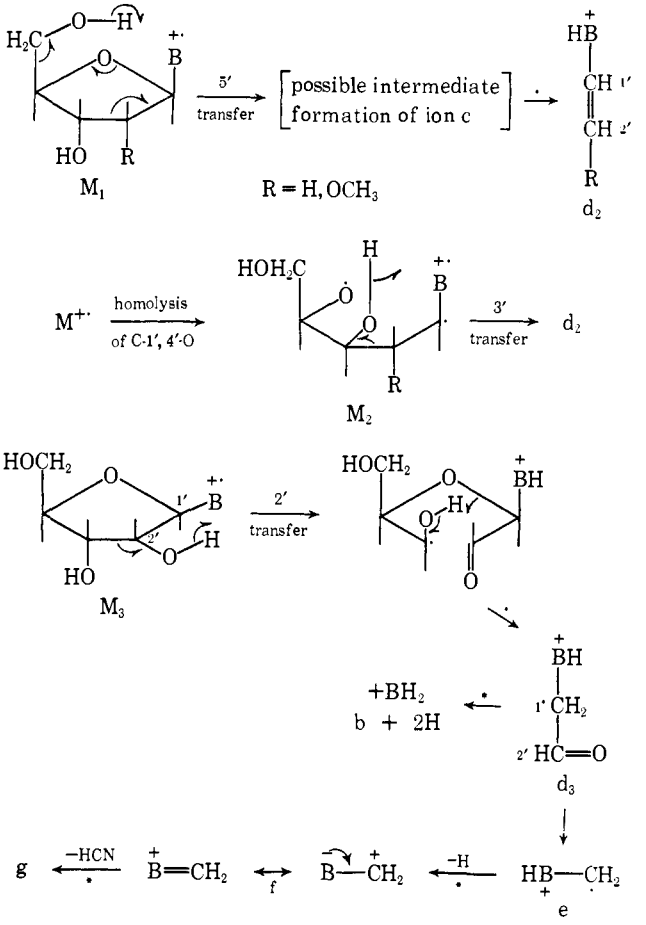


Figure 8. Mass spectrum of 9-β-D-psicofuranosyladenine (psicofuranine) (**25**).
Figure 9. Mass spectrum of N^6, N^6-dimethyladenosine (**3**).

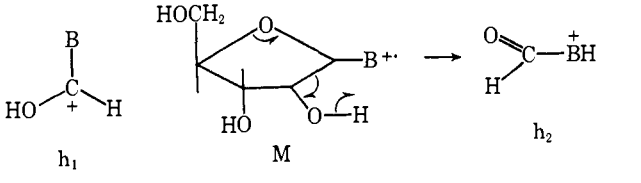


although we believe that one pathway may greatly predominate in any one compound. A number of mechanistic rationales can be formulated, involving charge localization in either the sugar or the base, and also involving transfer of an active hydrogen from any of the three possible positions in the sugar moiety. However, a choice of plausible ion structures for d must be governed in part by its further decomposition to ions e or b + 2. Based upon our original assumption of charge localization in the heterocyclic base, the following reactions are considered as the most mechanistically reasonable models for the formation of ion d.[54]

It is evident that d_3 is structurally better suited than d_2 for further decomposition to either e or b + 2. Since a free 2′-hydroxyl group is required for the transition $M_3 \rightarrow d_3$, the above models require compounds which bear no 2′-hydroxyl (*e.g.*, **2**, **14**) to proceed through d_2, from which expulsion of ketene to yield e would be mechanistically more difficult. As expected from these considerations, the abundances of ion e and its daughter product f (Table II) are markedly reduced in the mass spectra of compounds which lack the 2′-hydroxyl function (**2**, **14**, **18**, **19**, and **22**) compared with those in which it is present. As discussed in a previous section, labeling of active hydrogens by deuterium is an unsatisfactory technique with which to determine the precise labile hydrogen content of minor ions or those not cleanly separated in mass from other ion species. For this reason it is not presently possible to determine whether the process $d_3 \rightarrow$ e involves simple homolysis of the 1′–2′ bond without further hydrogen rearrangement. The same considerations hold for the identities of the two hydrogens in b + 2H, although in this case b + 2H may be generated from other precursors. In many cases further expulsion of HCN from f, marked by a metastable peak in a number of spectra, yields ion g, a minor but characteristic peak occurring at *m/e* 121 for adenine nucleosides.

(54) Eggers, Biedron, and Hawtrey[4] have proposed a structure for ion d in N^6,N^6-dimethyl-3′-deoxy-3′-aminoadenosine in which the rearranged hydrogen, whose source is not indicated, is bound to C-2′: +B=CHCH$_2$OH.

A characteristic and abundant ion in virtually all pentoside spectra occurs 30 mass units higher than the mass of the base (b), which contains the base, C-1′ and the pentose ether oxygen.[3] The utility of this ion (h) in reflecting structural changes at C-1′ is shown by the mass spectrum of psicofuranine (**25**), Figure 8, in which it occurs at *m/e* 194, 30 mass units higher than in adenosine (*m/e* 164, Figure 1) due to an additional CH_2O at C-1′. Similarly, replacement of the ring oxygen by sulfur in 4′-thioadenosine (**13**) results in a shift of ion h to *m/e* 180 (Table II). Originally assigned structure h_1, the rearranged hydrogen was thought to originate from the 2′-hydroxyl group, based on the low abundance of the ion in the spectra of 2′-deoxyribosides.[3] Evidence for participation of the 2′ function is supported by the present study; of the three deoxy isomers of adenosine (**2**, **16**, and **17**), only the 2′-deoxy compound exhibits a substantially reduced abundance of ion h, as seen in Table II. Similar comparisons hold for other compounds which are unable to provide a 2′-hydroxyl hydrogen: 2′,3′- and 2′,5′-dideoxyadenosine (**18** and **19**), and 2′-O-methyladenosine (**14**) *vs.* 3′-O-methyladenosine (**15**), the latter which bears a free 2′-hydroxyl group. Since this, as well as previous studies, shows transfer of a labile hydrogen in the formation of h, the reaction may be depicted as $M \rightarrow h_2$.

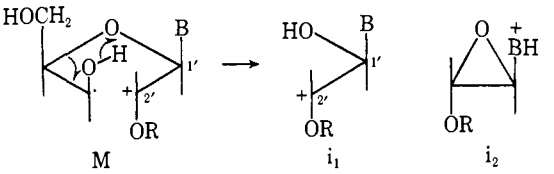


Derivation of h directly from the molecular ion is presumed, since no unambiguous metastable peaks were found in enough compounds to indicate a general pathway. An alternative pathway has been proposed by Eggers and coworkers in a study of N^6,N^6-dimethyl-3′-amino-3′deoxyadenosine (**26**), in which hydrogen is abstracted from the 3′ carbon, leading essentially to ion h_2. However, their mechanism must be precluded by the earlier labeling data.[3] Although the well-stabilized structure h_1 is also reasonable and has been widely accepted[6,19,25] two additional factors support the M → h_2 representation. Contrary to the conventional way of depicting riboside structures in which the 2′-hydroxyl is sterically inaccessible to the base, space-filling CPK molecular models[49] show that the two groups are in reasonably close proximity when the molecule is in the "*anti*" configuration. It is worthwhile to note two recent reports of pmr evidence for hydrogen bonding between the 2′-hydroxyl and N-3 of the base in 5′-mononucleotides.[55,56] While their results pertain to species in solution at relatively low temperatures, it is clear that direct interaction of the two groups (as in M → h_2) is easily possible without opening of the ribose ring. Metastable transitions representing the further decomposition of h by loss of CO are found in the spectra of most compounds studied, including adenosine. The double hydrogen rearrangement which would therefore be required for elimination of CO from h_1 indicates h_2 to be a more logical choice of ion structure.

Since the mechanism of formation of h_1 would probably depend on the availability of a ring heteroatom to act as a hydrogen acceptor, it is of interest to consider the behavior of 9β-(2α,3α-dihydroxy-4β-(hydroxymethyl)cyclopentyl)adenine,[57] an adenosine analog in which the ribose ether oxygen is replaced by a methylene group. Shealey and Clayton have reported[58] the major peaks from the mass spectrum of this compound, in which ion h (*m/e* 162) is the third most abundant ion (relative intensity 23%). While these data does not exclude the general existence of structure h_1 in pentoside mass spectra, it points to the alternative, and for the reasons outlined above, preferred formulation h_2.

The spectrum of 8-*d*-O,N-perdeuterio-2′-deoxyadenosine shows that neither of the two labile hydrogens in the sugar moiety is involved in the formation of ion h. The lower abundance of h in the spectra of 2′-deoxy compounds probably reflects in part the decreased tendency for abstraction of hydrogen from carbon relative to oxygen. A mechanism for formation of h in 2′-deoxy compounds cannot be proposed without further labeling studies. Participation of the hydrogen bound to C-2′ and *cis* with respect to the base along the 1′–2′ bond seems possible however since it comes in close proximity to the base when the base is rotated about the glycosidic bond.

A minor but structurally useful ion (i) which occurs widely in nucleoside mass spectra consists of the base plus 60 mass units of the sugar. Eggers and coworkers[4] identified the sugar fragment as containing C-1′ and 2′ and their attached oxygens, and proposed a mechanism involving 2′–3′ homolysis followed by transfer of a C-5′-hydrogen, arriving essentially at structure i_1 (R = H). Waller and collaborators have employed the same ion in the characterization of several 3′-modified adenosine analogs isolated from bacterial cultures.[19] The present study supports the general structure assignment of the ion but requires modification of the proposed mechanism. Ion i is found in the spectra of most pentoside analogs, including 5′-deoxyadenosine (**17**, Figure 6) and 2′-O-methyl adenosine (**14**, Figure 10), but is essentially absent in compounds which do not have active hydrogens at C-3′ (*e.g.*, **15** (<0.2%), **16**,[21] **18**), and in most 2′-deoxypentosides. However, the high-resolution spectrum of 2′-deoxyadenosine reveals an ion of the correct composition (base + C_2H_5O) for a deoxy form of i at *m/e* 178. The deuterium-labeled model d_4-**3** shows the inclusion of two deuterium atoms from the sugar moiety, therefore requiring transfer of a labile hydrogen in opposition to the mechanism proposed by Eggers.[4] It is evident from the above data that the transferred hydrogen originates at the 3′ heteroatom. A reasonable pathway can be formulated in which rearrangement to the ether oxygen is preceded by 2′,3′ cleavage with charge retention on C-2′, followed by collapse of the 4′-ether–oxygen bond. The resulting structure i_1 depends on a 2′ heteroatom for stabilization, thus accounting for



B = adenine
R = H; *m/e* 194
R = CH_3; *m/e* 208

its virtual absence in the spectra of 2′-deoxy compounds but presence in 2′-O-methyl nucleosides. Alternative mechanisms can be written in which rearrangement to the base occurs, resulting in a structure such as i_2 but which is considered less likely since it would less effectively explain the observed influence of a 2′ heteroatom.

Another small characteristic peak (j) is often observed 56 mass units higher than the base fragment in pentosides. The ion evidently contains C-1′,2′, and 3′ of the ribose skeleton, plus one heteroatom. Careful examination of a number of high-resolution spectra indicates that the mass of j does not shift consistently with methylation or heteroatom substitution (*e.g.*, sulfur in place of oxygen), hence its use for structure correlations in unknowns is limited. For instance ion j occurs at *m/e* 190 (relative intensity 1.0%) rather than *m/e* 206 in the spectrum of 3′-thioadenosine (**12**) indicating the absence of the 3′ heteroatom, while in the 3′-amino analog **26** the 3′ nitrogen is present (*m/e* 217, relative intensity 1.3%), and in 3′-O-methyladenosine (**15**) both possible values of j are found (*m/e* 190, 204, both 1% relative intensity).

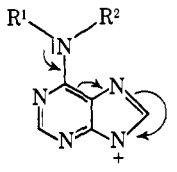
Perhaps the most generally characteristic ions in nucleoside mass spectra are those representing the base: b, b + H, and b + 2H. Since the masses of these fragments represent the base portion of the nucleo-

(55) M. P. Schweizer, A. D. Broom, P. O. P. Ts'o, and D. P. Hollis, *J. Amer. Chem. Soc.*, 90, 1042 (1968).
(56) S. S. Danyluk and F. E. Hruska, *Biochemistry*, 7, 1038 (1968).
(57) Y. F. Shealey and J. D. Clayton, *J. Amer. Chem. Soc.*, 88, 3885 (1966).
(58) Y. F. Shealey and J. D. Clayton, *ibid.*, 91, 3075 (1969).

side, their identification plays an important role in the structure determination of unknown molecules. Previous work has shown that the relative abundance of b + 2H is greater in the riboside than the corresponding 2′-deoxyriboside and is more pronounced in pyrimidine nucleosides.[3] Although such comparisons of b + H and b + 2H abundances can be made within a limited range of compounds, the present study indicates that conclusions should be drawn with caution since these ions evidently arise from multiple, unrelated paths. No metastable ion evidence was found to indicate a general fragmentation path leading to either b or b + H. Since these latter ions are abundant and differ in composition by one hydrogen, the path b + H → b appeared likely. Efforts were therefore made to detect a metastable peak corresponding to this transition in adenosine by the technique[59] of slightly increasing the ratio of accelerating voltage to electrostatic analyzer voltage. No transition was found even though the limit of detection of m* we estimated on our instrument to be $^1/_{5000}$ that of ion b. As previously discussed, b + 2H comes from at least two sources (ions d and h), but no metastable ion evidence was obtained to indicate further decomposition. The b + H ion, which formally corresponds to the molecular ion of the free base, sequentially eliminates three molecules of HCN in

$$\underset{\textbf{1},\ m/e\ 135}{b + H} \xrightarrow{-HCN} \underset{m/e\ 108}{k} \xrightarrow{-HCN} \underset{m/e\ 81}{l} \xrightarrow{-HCN} \underset{m/e\ 54}{m}$$

similar fashion to the behavior of adenine[7c] and 7-deazaadenine.[60] In adenine the first elimination involves retention of C-8 with loss of either C-2 or C-6, while about 65% of C-8 is eliminated in the second step.[61] Ion k, *m/e* 108 is a characteristic feature of virtually all adenine nucleoside mass spectra. The base fragment itself (b) is usually of lower abundance than its protonated counterparts. Ion b generally increases in per cent of total ionization when N^6 is substituted, due to inductive stabilization through the rings, as shown by **1**, **3**, **9**, and **11**.

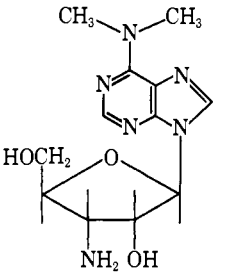


b

1, $R^1 = R^2 = H$; 0.4% rel int.
3, $R^1 = R^2 = CH_3$; 1.4% rel int.
9, $R^1 = H$; $R^2 = CH_3$; 2.3% rel int.
11, $R^1 = H$; $R^2 = C_5H_9$; 3.0% rel int.

Adenine nucleosides also exhibit a characteristic peak (n) at *m/e* 119. Its elemental composition ($C_5H_3N_4$[28]) corresponds to loss of the N^6 function from b + H, but in the absence of metastable peaks and extensive labeling data a detailed structure cannot be presently considered. As seen in Figure 9 similar ions (*m/e* 119–121, $C_3H_{3-5}N_4$), though possibly of different mechanistic origin, are found in mass spectra of N^6-substituted compounds.

Effects of Substitution at N^6 and O-2′. Alkylation at N^6 and O-2′ represents the most common forms of natural modification of adenosine. The mass spectra of purine nucleosides modified at these positions are structurally very informative, and are hence of interest. The present investigation has included the N-alkyl analogs **3**, **9**, **11**, and **26–28** and the O-methyl species **14**, **15**, **27**, **29**, and **30**.



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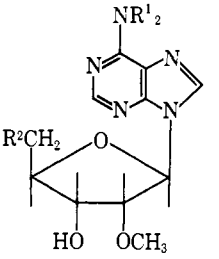
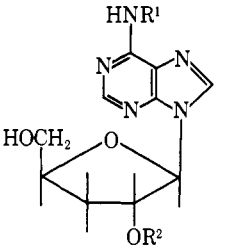
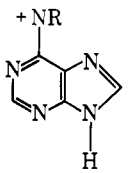


27, $R^1 = CH_3$; $R^2 = OH$
29, $R^1 = R^2 = H$

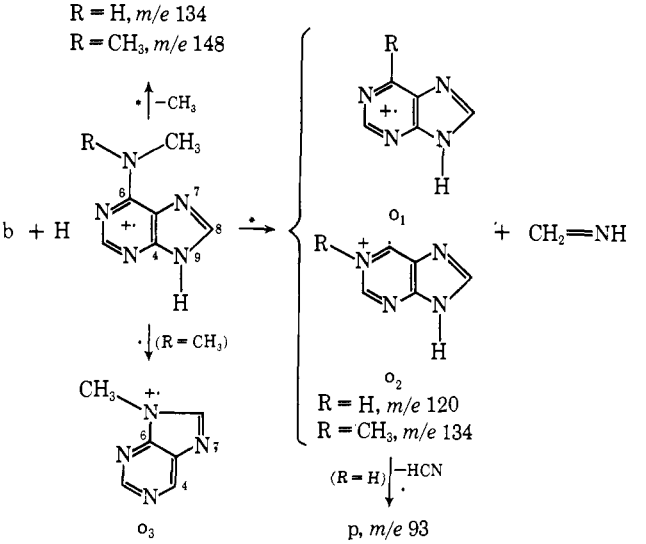


28, $R^1 = CH_3$; $R^2 = H$
30, $R^1 = H$; $R^2 = CH_3$



r

R = H, *m/e* 134
R = CH_3, *m/e* 148

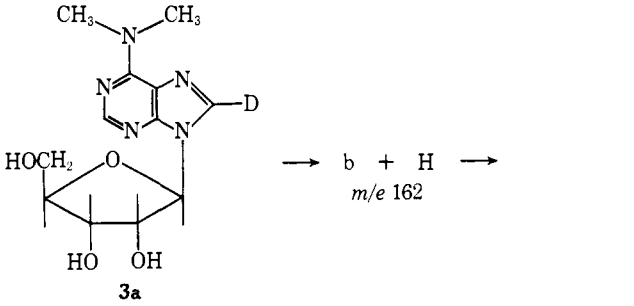


p, *m/e* 93

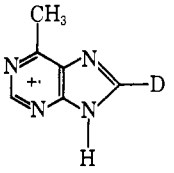
Methyl or dimethyl substitution at N^6 is uniquely characterized by elimination of CH_3N from the b + H ion[4] (ion o, Figure 9) in analogy to the same behavior of the molecular ion of the corresponding base.[7b,62]

(59) P. Schulze and A. L. Burlingame, *J. Chem. Phys.*, 49, 4870 (1968), and references therein.
(60) M. E. Smulson and R. J. Suhadolnik, *J. Biol. Chem.*, 242, 2872 (1967).
(61) J. L. Occolowitz, *Chem. Commun.*, 1226 (1968).
(62) Y. Rahamim, J. Sharvit, A. Mandelbaum, and M. Sprecher, *J. Org. Chem.*, 32, 3856 (1967).

In a study of N^6-methyladenine Shannon and Letham have suggested a mechanism corresponding to b + H → o_1, in which the rearranged hydrogen migrates to C-6.[7b] Methyl migration to N-1 in the formation of ion o in the case of N^6,N^6-dimethyladenine has been suggested by Rahamim and collaborators,[62] which would result in structure o_2. The latter assumption was based on the low tendency for the reaction to occur in the fragmentation of dimethylnaphthylamines. In a detailed consideration of the mass spectrum of the puromycin nucleoside (**26**) Eggers and coworkers[4] have proposed that elimination of CH_3N actually occurs from C-8 and N-9 of the imidazole ring, rather than from N^6, resulting in sequence b + H → o_3. Although disruption of the aromatic system to form o_3 at first seems unlikely, the resulting ion would in fact be well stabilized. To conclusively solve this point we examined the spectrum of 8-*d*-N^6,N^6-dimethyladenosine (**3a**), prepared by exchange of H-8 and the three active



3a



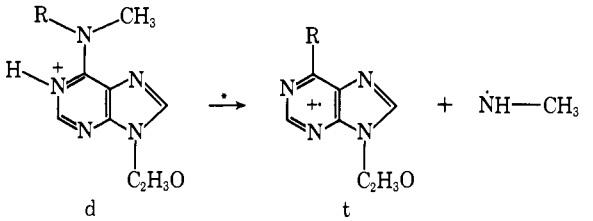
o, *m/e* 135

ribose hydrogens in warm D_2O–CH_3OD, followed by back exchange of the ribose deuteriums by cold CH_3OH. The deuterium content of ion o was 75% d_1 and 25% d_0, virtually identical with the molecular ion, indicating the absence of mechanism b + H → o_3. Expulsion of HCN from o in the monomethyl compounds produces ion p, *m/e* 93 (20.9% in **9**, 16.5% relative intensity in **28**). Although small peaks of correct composition ($C_4H_4N_3$) are present at *m/e* 93 in the spectra of the dimethyl compounds **3** (Figure 9) and **27** which correspond to analogous elimination of CH_3CN from o, the relationship was not confirmed by presence of appropriate metastable ions.

Further characteristic indication of methylation at N^6 is shown by a similar elimination of CH_3N from the b + 2H ion giving ion q, one mass unit higher than o. Ion q was of low abundance in the spectra of the dimethyl compounds studied (**3**, Figure 9, **27**), but more prominent in the monomethyl analogs **9** (32% relative intensity) and **28** (29% relative intensity).

Loss of an N^6-methyl group from b + H in N^6-dimethyl compounds produces the well-stabilized and diagnostic ion r (*m/e* 148, Figure 9). The same process is minor when the inductive influence of a second methyl group is absent (**9**, 2.8% relative intensity; **28**, 1.3% relative intensity).

As a pathway unique to N^6-methylated compounds, ion d (normally base + 44 mass units) eliminates CH_4N by rearrangement of an N^6-methyl group or hydrogen. The resulting ion (t, see Figure 9) will



R = H, *m/e* 162
R = CH_3, *m/e* 176

always occur at the same nominal mass as ion f in nucleosides unmodified at C-1′ and 2′. Since the two ion species differ by 24 mmass units (O *vs.* H_2N) they may be resolved at resolution $\Delta M/M = 7000$. In the spectra of **3** and **9** over 90% of the ion in question is due to the f component. In the case of the trimethyl compound **27** the ions occur at different masses due to substitution at O-2′: f at *m/e* 176 (5% relative intensity) and t at *m/e* 190 (6% relative intensity).

Mass spectrometry has found extensive use in the characterization of nucleosides and bases N substituted by a C_5 function, most of which exhibit cytokinin activity.[7b,12–16,22,25,26,63] The compound of principal interest has been 6-N-(3-methylbut-2-enylamino)-9-β-D-ribofuranosylpurine (**11**). The complete mass spectrum of **11** has been published by Hall, *et al.*,[12] and by Hecht and collaborators.[26] Detailed discussions of the identities and significance of the major fragment ions of **11** and closely related compounds have been previously presented,[7b,15,25,63b] and so will not be discussed here. Principal ions from the mass spectrum of **11** determined on an LKB instrument in our laboratory are listed in Table II for comparison with the other adenosine analogs listed.

One of the most interesting forms of nucleoside modification which occurs in nature is methylation in the ribose moiety at O-2′. These 2′-O-methyl nucleosides, often found in transfer RNA, are usually isolated as alkali stable dinucleotides and identified by their chromatographic or electrophoretic behavior.[64] Since, as discussed in this paper, the principal ions in nucleoside mass spectra consist of the intact base plus various portions of the sugar skeleton, mass spectrometry would *a priori* appear to hold considerable promise for the structure determination of 2′-O-methyl nucleosides. We have therefore examined the O-methylated analogs **14**, **15**, **27**, **29**, and **30**, and have found that 2′-O-methylation can be readily and uniquely characterized by mass spectrometry.

Mass spectra of 2′- and 3′-O-methyladenosine (**14** and **15**) are shown in Figures 10 and 11. The molecular ions of both compounds are found 14 mass units higher than in adenosine (Figure 1), and unlike the N-methylated derivatives examined in this study, show peaks at M − 15 due to loss of the O-2′- or -3′-methyl group (*m/e* 266),

(63) (a) N. J. Leonard and J. A. Deyrup, *J. Amer. Chem. Soc.*, **84**, 2148 (1962); (b) D. S. Letham, J. S. Shannon, and I. R. McDonald, *Proc. Chem. Soc.*, 230 (1964); (c) J. P. Helgeson and N. J. Leonard, *Proc. Nat. Acad. Sci. U. S.*, **56**, 60 (1966); (d) D. M. G. Martin and C. B. Reese, *J. Chem. Soc., C*, 1731 (1968); (e) G. Shaw, B. M. Smallwood, and D. V. Wilson, *ibid.*, 2999 (1968); (f) N. J. Leonard, S. M. Hecht, F. Skoog, and R. Y. Schmitz, *Proc. Nat. Acad. Sci. U. S.*, **59**, 15 (1968).

(64) For instance (a) M. W. Gray and B. G. Lane, *Biochim. Biophys. Acta*, **134**, 243 (1967); (b) S. Morisawa and E. Chargaff, *ibid.*, **169**, 285 (1968).

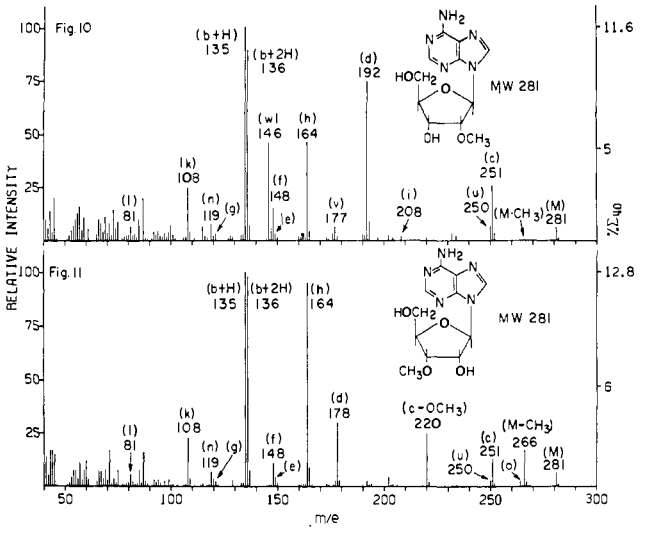
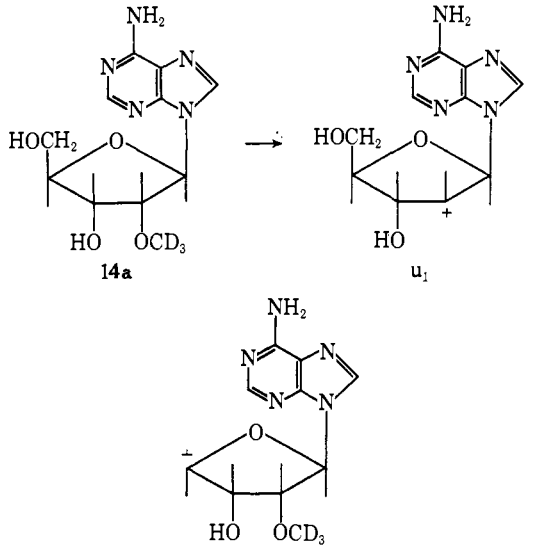


Figure 10. Mass spectrum of 2′-O-methyladenosine (**14**).
Figure 11. Mass spectrum of 3′-O-methyladenosine (**15**).

the tendency being greater in the latter compound. As expected from the foregoing discussions, certain ions diagnostic of an adenine nucleoside (b + H, b + 2H, e, f, h, k) are found at the same mass values as in adenosine. Loss of CH_3O from the molecular ion (to yield *m/e* 250) can occur from either the O-methyl moiety (ion u_1) or C-5′ (u_2). The deuterium-labeled analog 2′-O-(d_3-methyl)adenosine (**14a**) was therefore prepared and its mass spectrum examined. Loss of the ether radical (M − 34) was quantitatively preferred over the hydroxymethylene radical (M − 31), in favor of structure u_1.



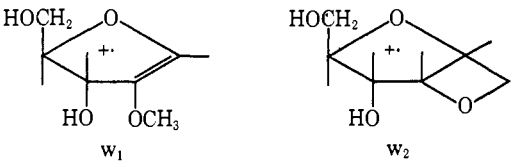
Unambiguous location of the methoxyl group on C-2′ is established by comparison of ions d and h. Since h occurs at *m/e* 164, indicating no additional substitution at C-1′, the shift of ion d from *m/e* 178 in adenosine (Figure 1) to *m/e* 192 in Figure 10 confirms methyl substitution in the C-2′ grouping. In 3′-O-methyladenosine (Figure 11) ions d and h occur at the usual values, while ion c (*m/e* 251) (which requires a free hydroxyl group at C-5′) is shifted 14 mass units higher than adenosine. In a decomposition step unique to 2′-O-methylated compounds, ion d further loses the O-2′-methyl group (*m** = $178^2/192$ = 165.0; 165.1 fd) to provide ion v, *m/e* 177 in Figure 10.

Ion h is reduced in abundance in the spectrum of the 2′-O-methyl compound **14** compared with that of **15**, in accordance with the preferential involvement of the 2′-O-hydrogen in the formation of that ion, as previously discussed. The spectra of 8-*d*-O,N-perdeuterio-**14** and **14a** show the predominant transfer of hydrogen from O-3′ or O-5′ to form ion h, but virtually none from the 2′-O-methyl group. As might be expected from their multiple paths of formation, b + H and b + 2H contain small amounts of the O-methyl hydrogens, less than 15% each.

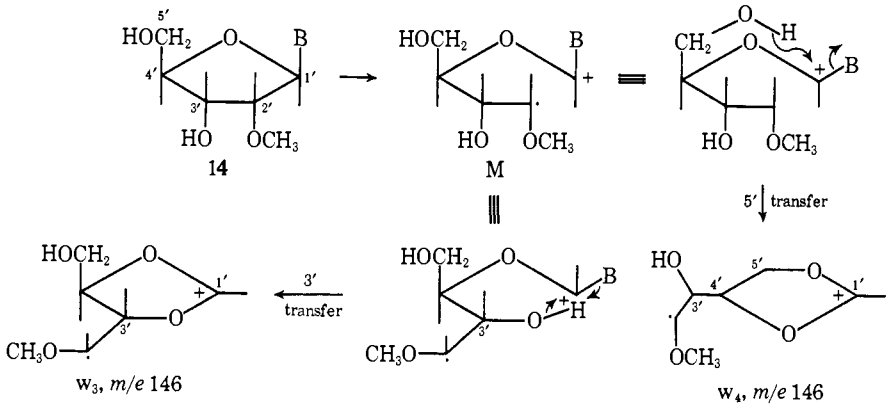
As shown in Figure 10 (**14**), an intense peak is observed at *m/e* 146 (ion w), which is essentially absent in Figure 11 (**15**). The high-resolution spectrum of **14** concludes the composition of w as $C_6H_{10}O_4$ (*m/e* 146.0579), one hydrogen less than the sugar fragment. This ion has been observed in the spectra of all 2′-O-methyl adenosine analogs included in the present study, as shown in Table III. Its occurrence also in the spectrum of a pyrimidine nucleoside, N^3-methyl-2′-O-methyluridine,[65] indicates the probability that ion w is characteristic and unique to all 2′-O-methylated nucleosides. The most logical ion structures would result from removal of hydrogen from either C-2′ (w_1) or the O-2′ methyl group (w_2). However, when **14a** and 8-*d*-O,N-perdeuterio-**14** were prepared to answer this question it was found that the missing hydrogen is predominantly labile (*i.e.*, from O-3′ or -5′), and essentially none is lost from the 2′-O-methyl function as would be required to support w_2. To determine whether a specific requirement exists for either the O-3′ or -5′ hydrogen the deoxy isomers **29** and **30** and their 8-*d*-O,N-perdeuterio analogs were prepared. As shown in Table III, ion w is formed in both cases, while a labile sugar hydrogen is still lost in the process. In attempting to formulate a reasonable mechanism for this interesting and diagnostically important process the two following factors must be considered. (1) The reaction depends upon the presence of an alkyl substituent on O-2′; since its hydrogens are not involved the role of the methyl group is apparently to provide inductive stabilization. (2) The resulting ion w must

Table III. Occurrence of Ion w (Sugar-H) in the Mass Spectra of 2′-O-Methyl Nucleosides

Compound	*m/e*	% RI	% Σ_{40}
2′-O-Methyladenosine (**14**)	146	45	1.5
N^6,N^6-Dimethyl-2′-O-methyladenosine (**27**)	146	15	1.8
2′-O-Methyl-5′-deoxyadenosine (**29**)	130	71	4.8
2′-O-Methyl-3′-deoxyadenosine (**30**)	130	41	4.5



(65) S. J. Shaw and J. A. McCloskey, unpublished results.



be very well stabilized, since the presence of an abundant sugar ion in purine nucleoside spectra is otherwise not observed due to the greater charge stabilizing ability of base-containing fragment ions. Taking these factors into account we envision as a plausible process initial rupture of the 1′–2′ bond with inductive stabilization of the 2′-radical site by the O-methyl group (**14** → M). Elimination of a neutral molecule of adenine follows transfer of hydrogen from either O-3′ or O-5′. The resulting species w_3 or w_4 are therefore secondary carbonium ions, highly stabilized by the unshared lone pairs of two adjacent ether oxygens.

Multiple methylation, as for example in N^6,N^6-dimethyl-2′-O-methyladenosine (**27**), results in a combination of diagnostically important ions which clearly indicate the sites of methylation. In the latter case the molecular ion is shifted 42 mass units (3 × CH_2) higher than adenosine to *m/e* 309 (see Table II). Ions b + H, b + 2H (*m/e* 163, 164), and h (*m/e* 192) reveal dimethylation on the base, which is further confirmed at N^6 by the transitions b + H and b + 2H → o (*m/e* 134, 100% relative intensity), and q (*m/e* 135, 14% relative intensity). The additional methyl group at O-2′ is confirmed by ions d and w (*m/e* 146, 15% relative intensity).

Effects Due to Changes in Hydroxyl Orientation in the Sugar. It can be reasonably expected that changes in the orientation of sugar hydroxyl groups will lead to variations in the abundances of some fragment ions, since mass spectrometry is often sensitive to such features in alicyclic compounds. Previous comparative examination of the mass spectra of the β-riboside, β-xyloside, and β-arabinoside of uracil revealed certain ion intensity variations, but detailed interpretations were not attempted.[3] The present study permits conclusions to be drawn from a larger number of closely related pairs of compounds: adenine 9α- and β-xyloside (**21**, **23**), adenine 9β-riboside (**1**) and -arabinoside (**31**), 2′-deoxy-α- and -β-adenosine (**22**, **2**), 9β-5′-deoxyadenosine (**17**) and adenine 9α-5′-deoxyxyloside (**20**), adenine allopyranoside and galactopyranoside (**32**, **33**). As seen in Table II and from the pairs shown in Figures 2, 3 and 4, 5, large and characteristic ion abundance variations exist, clearly permitting differentiation between each member of the pair. Attempts to correlate intensity variations with a particular change in structure—*i.e.*, to determine hydroxyl group orientation without reference compounds—were only moderately successful. From the mechanistic postulates presented earlier, the principal pathway for the formation of ion c (M − 5′-CH_2O) was proposed to require spatial proximity of the 5′ group and the base. From the data in Table II a strong correlation for this effect is evident. For those compounds in which the 5′-hydroxyl is "*trans*" to the base (**21** and **22**), only 2′-deoxy-α-adenosine (**22**) exhibits ion c, much reduced compared with its β anomer **2**, while in the spectrum of 9α-adenine xyloside it is absent, as previously discussed. From the data available thus far it seems appropriate to suggest that ion c is a strong—though not conclusive—indicator of the steric arrangement of C-5′. An obviously more direct approach to the question of hydroxyl group orientation would be through examination of the mass spectra of O-isopropylidene derivatives.[31]

An example of more extensive alterations in the sugar structure is offered by the hexose nucleosides 9-β-D-allopyranosyladenine (**32**, Figure 12) and 9-β-D-galactopyranosyladenine (**33**). From Figure 12 it can be seen that the pyranoside structure produces fragment ions of the same general types as the pentose furanosides, though from the differences in mass between M and the principal fragmentations it can readily be determined that the sugar is a hexose. The molecular ion is observed at *m/e* 297, or 30.0105 mass units (CH_2O) higher than in adenosine. The principal ions containing the intact base are present and are dominated by b + H (*m/e* 135) and h (*m/e* 164). The sugar fragment

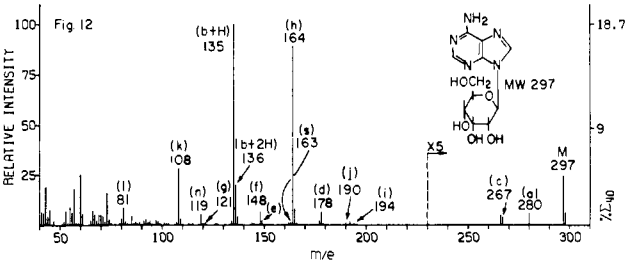
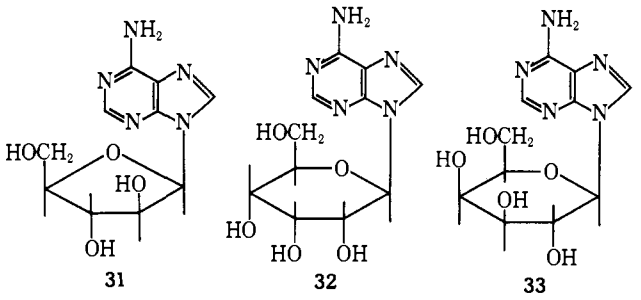


Figure 12. Mass spectrum of 9-β-D-allopyranosyladenine (**32**).



m/e 163 is of low abundance as is characteristic of purine nucleosides, but is easily recognized in the high-resolution spectrum ($C_6H_{11}O_5$ = *m/e* 163.0606). The presence of four hexose oxygen atoms in the sugar is shown by comparing elemental compositions of the molecular ion, *m/e* 163 and *m/e* 135 (b + H), obtained from exact mass measurements. Otherwise, details of the sugar structure are largely masked by the dominant base-containing ions. The mass spectra of pyrimidine hexosides may be expected to show a greater abundance of the sugar containing fragments. As seen in Table II, the principal effect of inverting the C-3′ and 4′ hydroxyls in the galactoside **33** is an increase in the abundance of b + 2H. Although it is tempting to attribute this increase to the greater steric availability of C-3′, 4′-hydroxyls in **33** *vs.* **32**, the probable multiple modes of formation of b + 2H dictate caution in such an interpretation.

Experimental Section

Melting points were measured on a Kofler hot-stage melting point apparatus. Low-resolution mass spectra were recorded on an LKB 9000 instrument; low-resolution spectra of O,N-perdeuterated compounds and all high-resolution spectra were obtained on a CEC 21-110B mass spectrometer. For high-resolution spectra photographic recording was employed; exact masses were measured of all ions greater than approximately 0.2% relative abundance. Ion source and direct inlet temperatures 210–250°; ionizing energy 70 eV.

Compounds from all sources were checked for purity by gas–liquid chromatography[30] and mass spectrometry[6] of their trimethylsilyl derivatives, and were further purified if necessary.

The following compounds were supplied by the Cancer Chemotherapy National Service Center of the National Institutes of Health: N^6-(3-methyl-2-butenyl)adenosine (**11**), 2′,3′-dideoxyadenosine (**18**), 3′-thioadenosine (**12**), 4′-thioadenosine (**13**), 2′,5′-dideoxyadenosine (**19**), 9-(5′-deoxy-α-xylofuranosyl)adenine (**20**), 9-β-xylofuranosyladenine (**21**), 9-(5′-deoxy-β-D-allofuranosyl)adenine (homoadenosine) (**24**), 9-β-psicofuranosyladenine (**25**), 9-β-D-allopyranosyladenine (**32**), and 9-β-D-galactopyranosyladenine (**33**). With the exception of the compounds which follow, all others were obtained from commercial or private sources.

2′-O-Methyladenosine (14) and **3′-O-methyladenosine (15)** were prepared by the action of diazomethane on adenosine.[66,67] Diazomethane, prepared from 1 g of N-nitrosomethylurea, was added to a solution of adenosine (1 g) in water (10 ml) with the latter solution maintained at 80°. After the disappearance of yellow color the solvents were removed and the residue retreated with diazomethane. After removal of solvents the residue was separated on Chromar sheets (Mallinckrodt Chemical Works) using $CHCl_3$–CH_3OH (4:1) into two spots. The more mobile component was isolated and subjected to anion exchange chromatography on a 21 × 1.5 cm Bio-Rad AG 1-2X(Cl^-) column (200–400 mesh). A good separation of **14** (eluted first) and **15** were obtained by elution with 12% CH_3OH in water. Fractions were collected by monitoring the elution at 260 mμ; **14** had a mp of 201° (lit.[68] 201–202°). Exact molecular mass of **14**, 281.1109; for **15**, 281.1090 (281.1124 required for $C_{11}H_{15}N_5O_4$ for both **14** and **15**).

N^6,N^6-Dimethyl-2′-O-methyladenosine (27), 2′-O-methyl-5′-deoxyadenosine (29), and 2′-O-methyl-3′-deoxyadenosine (30) were obtained by procedure similar to that above by O-methylation of **3, 17,** and **16,** respectively. Separation of methylated components in each case was not perfect; selected fractions from anion exchange chromatography were obtained for isolation with contamination from other methylated components. Insufficient material was available for recrystallization and melting points. Low-resolution mass spectra were free from contamination by starting material or any higher methylated nucleosides as judged by absence of appropriate molecular ions. Exact molecular mass of **27**, 309.1437 (309.1437 required for $C_{13}H_{19}N_5O_4$), of **29**, 265.1188; of **30**, 265.1137 (both require 265.1175 for $C_{11}H_{15}N_5O_3$). 2′-O-(d_3-Methyl)adenosine (**14a**) was prepared by carrying out the methylation in D_2O using CD_2N_2 which was generated by adding N-nitrosomethylurea to a 1:1 mixture of 1,2-dimethoxyethane and 40% KOH in D_2O. The resulting labeling pattern was 17% d_0, 3.6% d_1, 16% d_2, 58% d_3, 5.4% d_4.

5′-Deoxyadenosine (17).[69] 5′-O-*p*-Toluenesulfonyl 2′,3′-O-isopropylideneadenosine (0.5 g) was dissolved in acetic anhydride (8 ml) and heated for 10 min at 100° with NaI (0.6 g). The solution was cooled, $CHCl_3$ (20 ml) added, then extracted with saturated aqueous Na_2SO_3 followed by water. The product was purified by preparative tlc and extracted with methanol, giving N^6-acetyl-5′-iodo-5′-deoxy-2′,3′-O-isopropylideneadenosine,[70] which was dissolved in 4% 1 *N* HCl and hydrogenated overnight using 5% Pd–C as catalyst. The product (**17**) was isolated[71] as a white solid and purified on a Dowex 1-X2 (OH^-) resin column, and eluted with methanol–water (7:3): mp 212° (lit.[69] 210–212°). Exact molecular mass 251.1019 (251.1018 required for $C_{10}H_{13}N_5O_3$).

Acknowledgments. This work was supported by the National Institutes of Health (GM 13901) and the Robert A. Welch Foundation (Q-125). Computer services were provided by the Baylor College of Medicine Computer Facility (National Institutes of Health Grant No. FR 259) and the Common Research Computer Facility of the Texas Medical Center (FR 254). We are grateful to Mrs. J. Randolph and Miss P. Crain for technical assistance, and to Dr. Harry B. Wood, Jr., of the Cancer Chemotherapy National Service Center for his aid in obtaining numerous samples.

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