RESONANCE ENHANCED MULTIPHOTON IONIZATION OF NUCLEOSIDES BY USING PULSED-LASER DESORPTION IN SUPERSONIC BEAM MASS SPECTROMETRY

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(Received 28 June 1988)

ABSTRACT

Pulsed-laser desorption is used as a means of volatilizing nucleosides into the gas phase for entrainment into a supersonic jet expansion. These species are then studied in a time-of-flight mass spectrometer by resonance enhanced multiphoton ionization. Using this combination of techniques either relatively soft ionization or controlled fragmentation can be produced. The degree of molecular ion formation is shown to be relatively strong compared to other soft ionization methods such as FAB/MS, SIMS, FD or FI. In addition, the resulting fragmentation produces ions that are characteristic of the structure of the nucleoside. It is further demonstrated that such fragmentation can be used to discriminate between isomeric nucleosides and also between ribose and deoxyribose sugar groups.

INTRODUCTION

The study of nucleosides has presented a great challenge to mass spectrometry due to the polarity and thermal lability of these molecules. Most of the simple pyrimidine nucleosides and some of the purine nucleosides, such as adenosine, can be thermally vaporized and subsequently analyzed by electron impact (EI) or chemical ionization (CI) in a mass spectrometer [1–3]. However, the more polar nucleosides such as cytidine and guanosine, most complex natural nucleosides and all the nucleotides are not amenable to thermal vaporization without degradation [2]. This includes a significant class of nucleosides since in transfer RNA (tRNA) there are a variety of modified nucleosides present in addition to the four normally found in RNA. Modified nucleosides also occur in ribosomal RNAs, DNA and eukaryotic messenger RNA [4]. The function of these modified nucleosides

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is known to be important. More than sixty have been isolated and studied [5].

These thermally labile nucleosides and nucleotides have been studied by a number of techniques. Trimethylsilyl derivatization has been used as a means of volatilizing these compounds [6]. However, this process may result in chemical artifacts and uncertainties in the mass spectrum and may be inappropriate for a small amount of sample if conversion yields are low. In addition, EI (70 eV) of these compounds provides very extensive fragmentation with generally a very low relative abundance of the molecular ion peak, M^{+}, for identification [1,3,7–11]. More recently fast atom bombardment (FAB) has been used as a means of volatilizing and ionizing these compounds [12–15]. Using this method enhanced molecular ion peaks, MH^{+}, $(M - H)^{-}$, were observed in the positive and negative modes, respectively, although generally the base fragments BH_2^{+} and B^{-} were the dominant ions. In addition, FAB/CAD (collision-activated dissociation) in combination with MS/MS has been used to provide additional structural information and an extra degree of specificity [12]. Secondary ion mass spectrometry (SIMS) has been applied to modified nucleosides and the most intense peak corresponds to the base and only very minor molecular ion peaks were generally observed [16]. Field desorption (FD) and field ionization (FI) have also been applied to these compounds with relative soft ionization resulting, although BH^{+} was generally found to be the most abundant ion [17,18].

An alternative to these methods for the study of modified nucleosides is multiphoton ionization (MPI) [19]. MPI occurs when ionization follows the absorption of several photons when a molecule interacts with an intense visible or UV light source. When the laser frequency is tuned to a real intermediate electronic state, the MPI process is greatly enhanced and is known as resonance-enhanced multiphoton ionization (REMPI). When the laser is not tuned to a real state, the probability for MPI is nearly negligible. The most efficient version of this process is resonant two-photon ionization (R2PI), where one photon excites the molecule to an intermediate electronic state and a second photon induces ionization. The sum of the two photons must exceed the ionization potential for this process to occur efficiently. The advantage of R2PI is that, based on the wavelength chosen, one can produce relatively efficient soft ionization provided the excess energy of the two photons is relatively small compared to that required for ionization. This method has been used in previous work by our group [20–22,28] and that of Grotemeyer et al. [25–27,31] using a high-resolution reflectron TOF device to demonstrate relatively soft ionization of peptides [21,25–27], porphyrins [24], catecholamines [22] and other biological compounds [20–28] with either production of only the molecular ion or minimal fragmentation [20–27]. However, by increasing the energy of the photon or by increasing the power

of the laser, one can induce extensive fragmentation for structural analysis [29]. In the latter case the R2PI/MPI process may involve further absorption of many additional photons by the molecular ion, resulting in fragments as small as C^+ as the power density is further increased [29].

In this work we demonstrate the application of R2PI/MPI for structural analysis of modified nucleosides of adenosine, cytosine, guanosine and uridine. The volatilization process is accomplished using pulsed-laser desorption of neutrals with subsequent entrainment in a supersonic jet expansion of CO_2. Ionization and fragmentation are then produced using a second UV laser source operating at 266 nm. Thus, the desorption process is separated from the ionization process and the induced fragmentation can be controlled solely by the latter. In addition, the use of a supersonic jet expansion provides cooling of the internal modes of the molecules, thus minimizing the internal energy distribution and also providing translational cooling which results in enhanced resolution in a time-of-flight mass spectrometer.

EXPERIMENTAL

The supersonic beam time-of-flight mass spectrometer is similar to that used in previous experiments [22]. It consists of a differentially-pumped TOFMS mounted vertically in a stainless steel six-port cross pumped by a 6-in diffusion pump. The pulsed supersonic molecular beam expands into the ionization region of the TOF device and a laser beam perpendicular to both the supersonic jet and the flight tube ionizes the sample. The jet expansion is produced using a fast pulsed valve (R.M. Jordan Co., Grass Valley, CA) with a 55 μs pulse width at "choked flow". The nozzle to excitation distance is ~ 16 cm which permits a sufficient expansion distance to be in the free flow region of the CO_2 carrier gas.

Sample introduction was performed by using a pulsed CO_2 laser to desorb the compound of interest from the surface of an 1/8-in diameter machineable Macor ceramic rod (Corning Glass Works, New York). The samples were obtained from Sigma Chemical Co. and used without further purification. The rod was situated ca. 4 mm from the molecular beam axis and ca. 4 mm from the nozzle orifice. In this configuration only partial rovibronic cooling is observed following desorption and entrainment into the jet expansion and the absorptions remain broad enough to be resonant with the 266-nm laser radiation. The desorption occurs 4-mm downstream from the orifice in order to provide enhanced penetrability of the desorbed sample into the jet. If desorption is produced directly in front of the orifice the desorbed plume may not efficiently penetrate this region of the jet or else may cause shock waves which disrupt the jet flow. CO_2 is used as a

carrier gas since it provides a greater number of collisions further downstream than a simple monatomic carrier [30]. The result as demonstrated in previous work [20] is that extensive translational cooling was obtained as evidenced by the narrow ion peak widths obtained in the TOFMS. This indicates a correspondingly narrow energy spread in the beam resulting in a resolution up to ~ 600.

The nucleoside samples were deposited on the face of the rod by either wetting or dissolving the compound in benzene or methanol and coating the surface with the use of a spatula. In some cases the sample was dissolved in a glycerol matrix which provided a very stable pulse-to-pulse desorption over an extended period of time. In addition, recent work by Beavis et al. [31] has shown that the use of matrices can prevent thermal decomposition in the desorption process. The signal could be signal averaged over many hundreds of laser pulses using this method by carefully adjusting the laser power density on the surface. Typically, 10–100 μg of the sample was placed on the probe for study of the laser induced fragmentation patterns. The use of relatively thin layers of material prevents thermal decomposition in the desorption process as opposed to thicker samples which may absorb the IR radiation and cause heating in the sample. The desorption laser was an EXC-1 Quanta-Ray CO_2 laser. A Scientech 365 meter was used for measurement of the beam energy, and based upon the beam profile [22], the power density of the beam was estimated to be a maximum of 7×10^6 W cm^{-2} on the surface of the rod. The IR beam was softly focused with a 10-cm focal length biconvex germanium lens to a ~ 2–3 mm spot for desorption, although this was adjusted for each case to optimize our results. The key point here is that a rather low power CO_2 laser (40-mJ output maximum) can be used to generate neutral species by desorption as opposed to present laser desorption mass spectrometry (LDMS) experiments where high-power lasers (~ 0.5 J) may be needed to generate significant numbers of ions. Also in our experiments the CO_2 laser focus may affect the results obtained but there is a large margin of play in this parameter to produce the desired results. Over a range of 1×10^6 to 2×10^7 W cm^{-2} the desorption was found not to affect the laser MPI results obtained.

The ionization source was the 4th harmonic of the Quanta-Ray DCR-2A Nd:YAG laser at 266 nm. The laser energy was monitored by a power meter and was held constant at ~ 10 mJ throughout the fragmentation patterns induced in the nucleosides studied herein. This corresponds to a power density of ~ 8×10^6 W cm^{-2}. This relatively high power density was used to induce extensive fragmentation specifically for structural analysis. In order to decrease the degree of fragmentation the power was reduced typically to ~ $1–2 \times 10^6$ W cm^{-2}. In most experiments the ionizing laser beam was collimated by a positive–negative lens combination to a beam 2–3

mm in diameter. The actual sequence of events was controlled by several delay generators where the pulsed CO_2 laser was fired first to produce desorption followed by the pulsing of the valve. The two events were synchronized in time so that the desorbed plume was entrapped into the jet expansion of CO_2 and carried into the acceleration region of the TOFMS. The flight time of the jet from the pulsed valve to this region was about 300 μs and the laser was set to pulse as the gas pulse arrived. Laser R2PI was produced and a LeCroy 9400 digital oscilloscope was used to record the mass spectrum.

RESULTS AND DISCUSSION

In our initial studies the laser-induced ionization of the various bases at 266 nm was re-examined. As shown in previous studies [20,22], soft ionization mass spectra of adenine and cytosine were observed using laser desorption followed by R2PI at modest laser energy ($\sim 10^6$ W cm^{-2}). In addition, ionization of guanine was also observed at 266 nm due to the optimization of the desorption/entrainment of sample into the jet over that attained in previous work. Under the conditions of the experiment only the molecular ion with no fragmentation could be produced. However, even under the conditions of the present experiment uracil could not be ionized. This can be attributed to the high ionization potential of uracil (IP = 9.50 eV) [32]. The sum of the energy of the two photons at 266 nm (9.2 eV) is not sufficient to reach the ionization limit of this molecule.

By increasing the laser power to $\sim 4 \times 10^6$ W cm^{-2} at 266 nm, characteristic fragments can be induced in these bases. This is illustrated for adenine and guanine in Fig. 1. The most abundant ion is still $M^{+\cdot}$, and fragments at M $-$ 16 (m/z 119 for adenine and m/z 135 for guanine) result from the loss of the amino group while M $-$ 17 corresponding to loss of NH_3 is observed for guanine. In the case of adenine, a second fragment at m/z 108 is due to expulsion of HCN. For guanine, the fragment at m/z 109 results from elimination of H_2NCN from the molecular ion. As the laser power is increased further, a variety of fragments at below m/z 100 are observed. The mass spectra of these compounds have been studied by electron impact mass spectrometry [7–9]. Since laser R2PI/MPI involves the initial production and subsequent fragmentation of $M^{+\cdot}$, it might be expected that the observed fragmentation patterns would be similar to EI. One advantage of R2PI over EI is that the near UV laser radiation is transparent to the CO_2 carrier, and will only directly ionize the analyte. Thus, R2PI avoids the carrier background that would be generated by EI. A second advantage of R2PI is that the efficiency of the ionization process changes by less than an order of magnitude from the conditions required to generate soft ionization
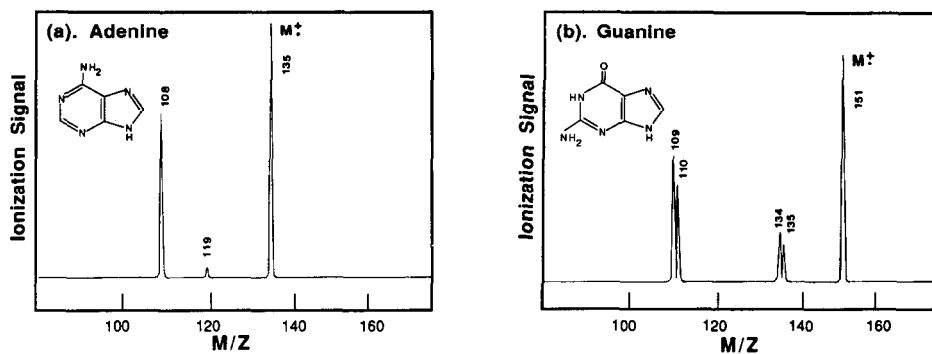
Fig. 1. Laser-induced mass spectra of free bases: (a) adenine; (b) guanine at 266 nm. The laser ionization power density was 4×10^6 W cm^{-2}.

to that for extensive fragmentation of these nucleosides. Generally a significant loss in sensitivity is observed in EI as the electron energy is decreased to produce a soft ionization mass spectrum.

The laser desorption/laser R2PI method was next used to study adenosine, guanosine and cytosine as well as their various modified nucleosides as shown in Tables 1–3. These results were obtained at $\lambda = 266$ nm and $P = 8 \times 10^6$ W cm^{-1}. The salient feature of the mass spectra observed under these conditions is an intense molecular ion peak without the presence of cationization. In addition, there is generally one principal fragment observed due to bond cleavage at the sugar moiety. This fragment denoted BH$^+$ (B is the molecular weight of the free base minus 1) corresponds to an ion formed from the base portion of the nucleoside with transfer of one hydrogen atom from the sugar moiety. This process is also observed in EI mass spectra of these compounds [1,3]. The molecular ion peak, along with the characteristic BH$^+$ peak, can be used to distinguish between ribose and deoxyribose sugars in the nucleoside.

In addition, two characteristic fragments due to cleavage of the sugar moiety are observed which are denoted as S_1 and S_2, respectively, as shown in Fig. 2. These fragment ions are particularly useful for structural analysis and confer the ability to easily pinpoint whether there is modification on the 2'-oxygen of a nucleoside. As Table 1 indicates, the mass spectrum of 2'-deoxyadenosine shows a strong molecular ion peak m/z 251 and a fragment BH$^+$, m/z 135. The mass difference between M$^{+\cdot}$ and BH$^+$ suggests that it is a deoxyribose. Two fragments, m/z 162 and m/z 164, corresponding to S_1 and S_2 notation, respectively, can be used to locate the deoxy position, i.e., the 2'-position in this case. Or, for example, in 3'-*o*-methyladenosine, S_1 and S_2 fragments indicate that there is no methyl substitution on the 2'-oxygen. Consequently, the methyl group must be on

TABLE 1

Mass spectral fragmentation patterns of adenosine analogs [a]

Compound	m/z (rel. int.%)						
	M	S_1	S_2	BH	119	108	Others
Adenosine	267(43)	178(19)	164(33)	135(100)	119(29)	108(38)	
2'-Deoxyadenosine	251(42)	162(15)	164(23)	135(100)	119(17)	108(45)	
N^6-Methyladenosine	281(51)	192(17)	178(24)	149(100)	119(40)	108(24)	
N^6-Methyldeoxyadenosine	265(40)	176(17)	178(18)	149(100)	119(30)	108(23)	
1-Methyladenosine	281(42)	192(16)	178(18)	149(100)			148(38),134(69),121(20)
N^6,N^6-Dimethyladenosine	295(100)	206(7)	192(14)	163(50)	119(16)	108(5)	147(11)
3'-o-Methyladenosine	281(50)	178(5)	164(32)	135(100)	119(16)	108(19)	132(21),105(39)
1,N^6-Ethenoadenosine	291(99)			159(100)			134(10)
2,6-Diaminopurine-2'-deoxyribosine	266(61)	177(7)		150(100)		108(18)	
N^6-Furfuryladenosine	347(88)			215(100)	119(62)	108(50)	
N^6-(2-Isopentenyl)adenosine	335(100)			203(47)	119(73)	108(39)	188(43),160(34),135(46)
Puromycin aminonucleoside	294(77)	206(9)	192(35)	163(100)	119(23)	108(77)	222(9),148(38),134(47), 121(18),114(6)

[a] Laser ionization is achieved at 266 nm and a power density of $\sim 8 \times 10^6$ W cm^{-2}.

TABLE 2

Mass spectral fragmentation patterns of guanosine analogs [a]

Compound	m/z (rel. int.%)						
	M	S_1	S_2	BH	135	109	Others
Guanosine	283(34)	194(15)	180(10)	151(100)	135(44)	109(20)	
2'-Deoxyguanosine	267(18)	178(14)	180(5)	151(100)	135(25)	109(20)	
7-Methylguanosine	297(12)	208(6)	194(6)	165(100)			149(47), 123(45)
1-Methylguanosine	297(18)			165(100)			
N^2-Methylguanosine	297(73)	208(5)	194(5)	165(100)	135(20)	109(33)	
3'-o-Methylguanosine	297(63)	194(10)	180(10)	151(100)	135(31)	109(27)	

[a] Laser ionization is achieved at 266 nm and a power density of $\sim 8 \times 10^6$ W cm^{-2}.

TABLE 3

Mass spectral fragmentation patterns of cytidine analogs [a]

Compound	m/z (rel. int. %)				
	M	S_1	S_2	BH	Others
Cytidine	243(31)	154(26)	140(31)	111(100)	
2'-Deoxycytidine	227(23)	138(18)	140(32)	111(100)	
2'-o-Methylcytidine	257(32)			111(100)	
5-Methylcytidine	257(23)	168(8)	154(18)	125(100)	109(17)
3,N^4-Ethenocytidine	267(31)	178(18)		135(100)	105(32)

[a] Laser ionization is achieved at 266 nm and a power density of $\sim 8 \times 10^6$ W cm^{-2}.

either the 3'-oxygen or 5'-oxygen. The formation of S_1 and S_2 in R2PI/MPI appears to be similar to EI in which an intramolecular rearrangement of the H from the sugar to the base occurs during fragmentation [1,3]. These S_1
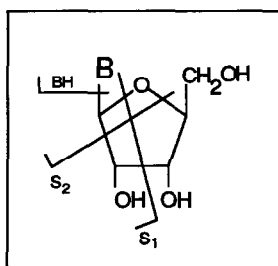

Fig. 2. Major ions observed for the nucleosides using the laser desorption/laser ionization technique at 266 nm: (1) molecular ion M$^+$; (2) base plus one hydrogen BH$^+$; (3) base and parts of sugar plus one hydrogen S_1^+ and S_2^+; (4) fragments from BH$^+$.

and S_2 fragments are easy to detect using R2PI/MPI as opposed to FAB where their presence may be obscured due to matrix background [12].

There are several other characteristic fragments observed in laser R2PI/MPI. In the case of the adenosine nucleosides, two fragments at m/z 108 and m/z 119 are observed due to fragmentation of the base as is also observed in EI [3]. In the guanosine nucleosides, characteristic peaks are likewise observed at m/z 109 and m/z 135. However, other fragment ions observed in EI such as those involving –OH and H_2O elimination are not observed. For the cytidine analogs (Table 3), no fragments from BH^+ are observed. Indeed, even for the free base cytosine we can only obtain the molecular ion with no fragments at the same power density, i.e., 8×10^6 W cm^{-2}. This indicates that the cytosine molecular ion or equivalently BH^+ from the cytidine is quite stable when produced by laser R2PI at 266 nm. It should be noted that the observed fragmentation patterns were due to laser R2PI/MPI and not the laser desorption process. This was checked by varying the desorption laser power over a range of 1×10^6 to 2×10^7 W cm^{-2} with no apparent change in the relative intensity of the fragments at constant laser ionization energy.

One of the advantages of using lasers as an ionization source for mass spectrometry over other soft ionization techniques is the ability to control the fragmentation pattern of the mass spectrum by simply changing the ionization laser power density or using different wavelengths. The soft ionization can be used for unique detection of components of interest in a complicated mixture based on the exact molecular weight, while extensive fragmentation is necessary for the structural elucidation of an unknown. The dependence of the fragmentation on the laser power density was studied over the range 1×10^6 W cm^{-2} to 8×10^6 W cm^{-2} and is shown for N^6,N^6-dimethyladenosine in Fig. 3. At 1×10^6 W cm^{-2} only the molecular ion is obtained with no fragmentation (see Fig. 3d). However, in most of the cases studied herein a BH^+ fragment generally accompanies $M^{+\cdot}$ even at the lowest energy used. As the laser power is increased, extensive fragmentation is obtained. The mass spectra in Fig. 3 are drawn to the same scale. Note that as the laser power is decreased in order to minimize fragmentation, a loss of signal intensity and thus sensitivity also results. However, by adjusting the laser power the fragmentation can be selectively controlled. In Fig. 3a extensive fragmentation of N^6,N^6-dimethyladenosine is obtained at 8×10^6 W cm^{-2}. The molecular ion is the dominant peak in the mass spectrum accompanied by the base, BH^+, fragment at m/z 163. The S_1 and S_2 fragments are also present in relatively low abundance at m/z 206 and m/z 192, respectively. The m/z 148 ion is the result of CH_3 expulsion from BH^+. Further fragmentation of BH^+ also gives the ions m/z 134 $(BH - 29)^+$ and m/z 121 $(BH - NCNH_2)^+$. In addition m/z 119 and m/z 108, which are characteristic of the adenine base, are also observed.
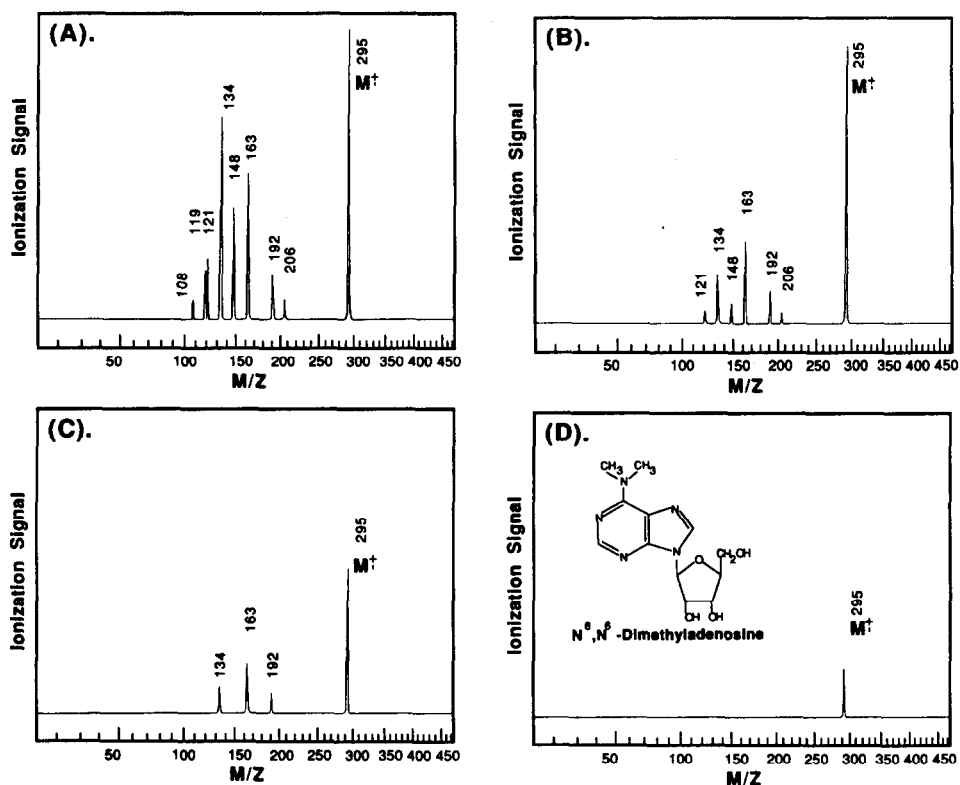
Fig. 3. Mass spectra of N^6,N^6-dimethyladenosine obtained by the laser desorption/laser ionization technique at 266 nm. The ionization laser power density was: (A) 8×10^6 W cm^{-2}; (B) 4×10^6 W cm^{-2}; (C) 2×10^6 W cm^{-2}; and (D) 1×10^6 W cm^{-2}. These spectra are signal averaged over 100 laser pulses.

It should be noted that uridine and many of its analog nucleosides cannot be ionized by R2PI/MPI at 266 nm under the conditions of this experiment. However, if a strong electron-releasing group such as $-NH_2$ is attached to the base, then ionization may be observed as shown in Fig. 4 for aminouridine. The strong electron releasing substituent not only reduces the ionization potential of the molecule but also enhances the cross-section for absorption at 266 nm. Also, the addition of an absorbing center such as the monomethoxytrityl group will enhance ionization in these uridine compounds. This is demonstrated in Fig. 5 for 5'-o-monomethoxytrityluridine which is easily ionized at 266 nm. 5'-Monomethoxytrityl-substituted adenosine, guanosine and cytosine have also been studied. We have found that in general the 5'-monomethoxytrityl-substituted nucleosides are about ten times more sensitive than the unsubstituted nucleosides. This demonstrates that the R2PI active center such as the 5'-monomethoxytrityl group can be used
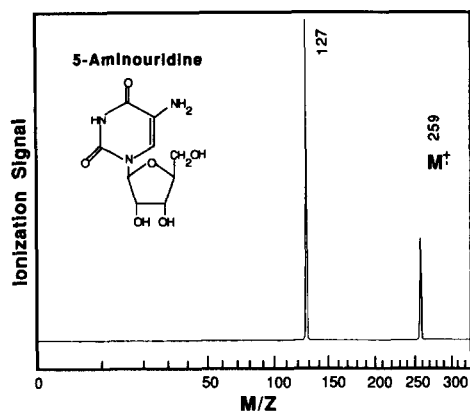
Fig. 4. Laser-induced mass spectrum of 5-aminouridine at 266 nm. The ionization laser power density was 4×10^6 W cm^{-2}.

to enhance the ionization efficiency. Notice that the mass spectrum of 5′-*o*-monomethoxytrityluridine (Fig. 5) is complicated by the presence of the fragments from the 5′-*o*-monomethoxytrityl group.
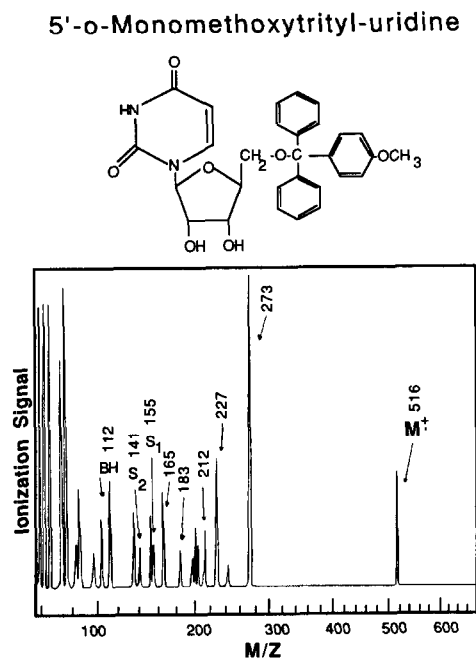

Fig. 5. Laser-induced mass spectrum of 5′-monomethoxytrityl-uridine at 266 nm. The ionization laser power density was 4×10^6 W cm^{-2}.
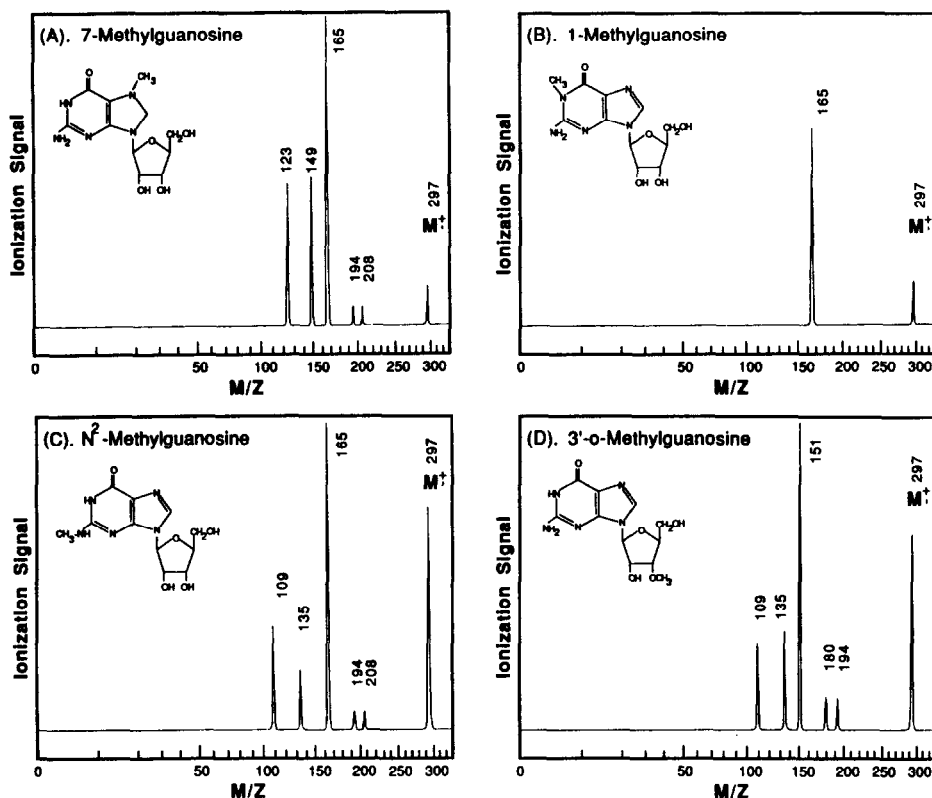
Fig. 6. Mass spectra of four isomers of methyl-substituted guanosine at 266 nm ($P = 8 \times 10^6$ W cm^{-2}): (A) 7-methylguanosine; (B) 1-methylguanosine; (C) N^2-methylguanosine; (D) 3'-o-methylguanosine.

An important aspect of the laser-induced R2PI/MPI process is the ability to induce fragmentation by which isomeric nucleosides can be distinguished. This is demonstrated in Fig. 6a–d for the four isomers of methyl-substituted guanosine at 266 nm ($P = 8 \times 10^6$ W cm^{-2}). The m/z 165 peak (Fig. 6a–c) is the BH$^+$ ion from which one can conclude that the methyl group is located on the base. The m/z 151 peak (Fig. 6d) observed in the mass spectrum of 3'-o-methylguanosine indicates that the methyl group is in the sugar portion of the nucleoside. Other fragments can be used to distinguish the position of the methyl group in the base. In the case of 7-methylguano-sine, m/z 149 is due to loss of the –NH$_2$ group from BH$^+$ while m/z 123 results from expulsion of cyanamide H$_2$NCN from BH$^+$. For 1-methyl-guanosine, no fragments from BH$^+$ are observed at a laser power of 8×10^6 W cm^{-2}. The m/z 135 ion observed for N^2-methylguanosine is due to loss of CH$_3$–NH from BH$^+$ and m/z 109 is due to loss of CH$_3$–NHCN from

BH^+. At the same laser energy these fragments are not observed for 7-methylguanosine and 1-methylguanosine.

The detection limit for several of these nucleosides was studied. A series of successive dilutions were prepared and deposited on the desorption surface. Laser desorption of these samples into the supersonic jet was performed followed by R2PI/MPI at 266 nm ($P = 1 \times 10^7$ W cm^{-2}). In general the detection limits for these nucleosides (molecular ion signal was monitored) are in the range 100–500 ng. For example, the detection limit of N^6, N^6-dimethyladenosine is about 185 ng with $S/N = 2$ and about 250 ng for 3'-o-methylguanosine. In most of these cases the ionization potential (IP) appears to be sufficiently low for R2PI to proceed, i.e., adenine IP = 8.44 eV, guanine IP = 8.24 eV [32]. However, it appears that these molecules have a rather low cross-section for absorption. From the gas phase UV spectrum of adenine and the UV spectra of nucleosides in solution, we conclude that a strong absorption occurs generally at 240–260 nm for these nucleosides in the gas phase. We expect the use of laser radiation in this region will enhance the sensitivity of this technique.

In conclusion, pulsed-laser desorption can be used to volatilize polar nucleosides that are not readily amenable to thermal vaporization. Resonance-enhanced multiphoton ionization then serves as a means to softly ionize those species where an $M^{+\cdot}$ ion of relatively high abundance results, compared to other methods that have been used to examine these compounds. In addition, by increasing the laser power fragmentation can be produced which reflects the structure of the molecule. The characteristic S_1 and S_2 fragments in particular can provide structural information regarding substitutions on the sugar moiety. In addition, at equivalent laser power, the fragmentation produced by R2PI/MPI can be used to uniquely distinguish between isomeric substituted nucleosides.

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