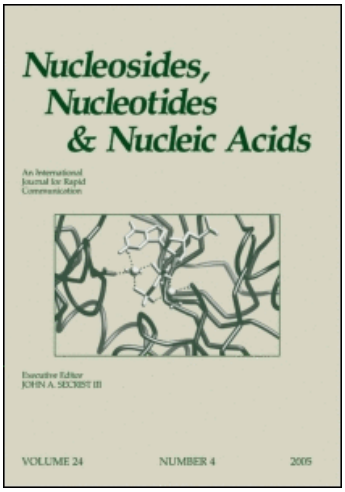

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Esmans, E. L. , Belmans, M. , Vrijens, I. , Luyten, Y. , Alderweireldt, F. C. , Wotring, L. L. and Townsend, L. B.(1987) 'Analysis of Substituted Pyridine-C-Nucleosides by Direct Liquid Introduction Liquid Chromatography/Mass Spectrometry', Nucleosides, Nucleotides and Nucleic Acids, 6: 5, 865 — 876
To link to this Article: DOI: 10.1080/15257778708073433
URL: http://dx.doi.org/10.1080/15257778708073433

NUCLEOSIDES & NUCLEOTIDES, 6(5), 865-876 (1987)

ANALYSIS OF SUBSTITUTED PYRIDINE-C-NUCLEOSIDES BY DIRECT LIQUID INTRODUCTION LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY.

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Abstract:
A method was elaborated for the analysis of a few original pyridine-C-nucleosides via microbore DLI/LC-MS. The compounds were analyzed on a 10RP8 column (25 cm x 1 mm) using a number of 0.01 M $HCOONH_4/CH_3OH$ mixtures as eluant. Under appropriate LC-MS conditions, both α- and β-anomers were separated and identified. All nucleosides were characterized by the protonated molecular ion $[MH]^+$, $[B+30]^+$ and $[B+44]^+$-fragment ions. Assignment of the α,β-configuration at C_1' was done with the aid of ^{13}C-NMR. From the DLI/LC-MS data, a semi-preparative HPLC-method was developed to purify the pyridine-C-nucleosides prior to biological evaluation.

I. Introduction.

In the past, high pressure liquid chromatography (HPLC) has emerged as a useful technique for the separation and purification of nucleosides[1].

Since the reliability of the biological data obtained for synthetic nucleoside analogs in different test systems (L-1210 mouse leukemia, coxsackievirus, poliovirus, e.g.) is highly dependent upon the purity of the compounds submitted, considerable effort has been expended in the development of sensitive and powerful analytical techniques. A technique which meets

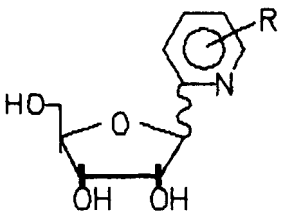
the desired requirements is combined liquid chromatography-mass spectrometry (LC-MS)[2,3,4].

In this paper, we wish to discuss the results of this technique for a series of substituted pyridine-C-nucleosides, which in turn is part of a programme aimed at the development of antitumor and/or antiviral drugs.

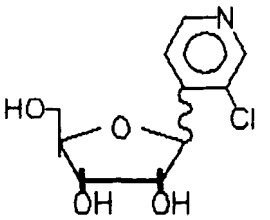
II. RESULTS AND DISCUSSION.

A series of substituted pyridine-C-nucleosides (I to XI) was synthesized using organo-lithium intermediates of pyridine derivatives and 2,4;3,5-di-O-benzylidene-D-ribose[5,6,7].

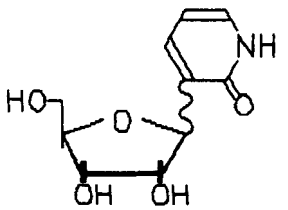
In order to obtain rapid and reliable information as well on structure as on purity, the crude reaction mixtures were investigated by DLI/LC-MS in a configuration described elsewhere[3].



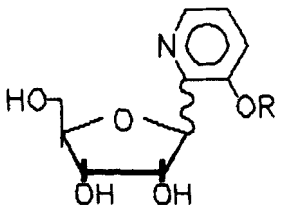
I : R=H ; II : R=3-CH_3 ;
III : R=4-CH_3 ; IV : R=5-CH_3 ;
V : R=6-CH_3



VI



VII



VIII : R=CH_3 ; IX : R=C_2H_5 ;
X : R=C_3H_7 ; XI : R=C_4H_9

COMPOUNDS

Compounds I to VII were analyzed on a microbore 10RP8 column (25 cm x 1 mm I.D.) using 85% 0.01 M $HCOONH_4$/15% CH_3OH as the eluant, at a flow-rate of 80 μL/min. A chromatogram, which can be considered representative for the analysis of compounds I to V, is depicted in FIG. 1. The data for the isomeric compounds I to IV are summarized in TABLE 1.

As can be seen in FIG. 1, 6-methyl-2-(D-ribofuranosyl)-pyridine (V) was located with the aid of the reconstructed ion chromatogram for the protonated molecular ion $[MH]^+$ at m/z = 226. Because two chromatographic peaks, with respective retention times of 7.15 and 12.33 min, were found to respond at this m/z-value, it was concluded that the separation of the α-anomer from the β-anomer had been accomplished. The mass spectra of these compounds were all characterized by a protonated molecular ion $[MH]^+$ which was in all cases the base peak. Also in both components the rearrangement ions at $[B+30]^+$ and $[B+44]^+$ were detected giving information about the structure of the base moiety (TABLE 1).

However, due to the simularities observed in the mass spectra of both anomers (FIGS. 2-3), an assignment of the absolute configuration (α or β) remained uncertain.

Therefore an experiment was set up separating a mixture of α- and β-adenosine under analogous conditions, followed by the injection of pure β-adenosine (FIG. 5).

From these results it could be concluded that α-adenosine had the lowest k'-value. This was an indication that in the series I-XI, the α-anomer would elute prior to the β-anomer. Furthermore, since in the ^{13}C-NMR spectra, the signals of the carbon atoms of the α- and β-anomers could be unequivocally assigned, the ^{13}C-NMR peak integration data could be compared to the corresponding chromatographic peak areas calculated from the LC-MS runs (TABLE 2). From these experiments there is no doubt that the α-anomer is the isomer with the lowest k'-value.

Together with the 6-methyl-C-nucleosides described above,

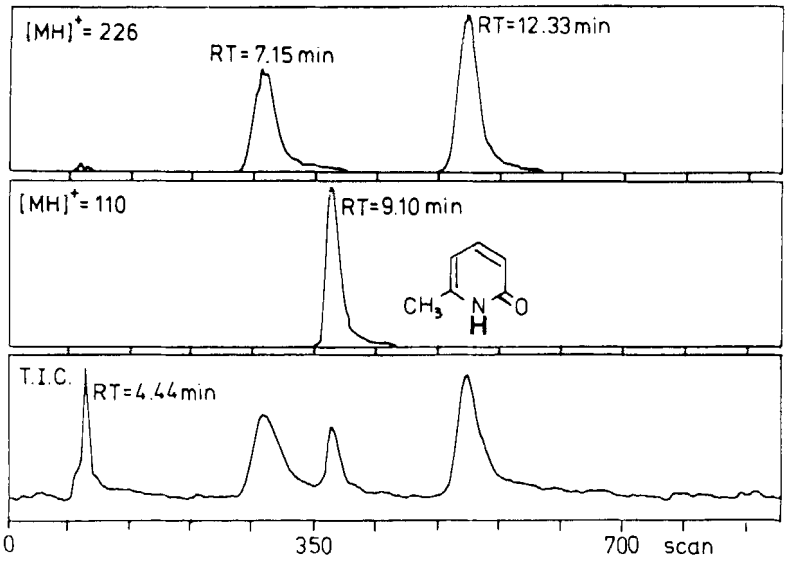


FIG. 1 : Microbore DLI/LC-MS of a crude mixture of 2-(D-ribofuranosyl)-6-methylpyridine (V). Eluant : 85% 0.01 M $HCOONH_4$/15% CH_3OH ; flow-rate : 80 µL/min ; column : Microbore 10RP8 (25 cm x 1 mm I.D.). T_{source} = 190°C ; $T_{desolvation\ chamber}$ = 210°C.

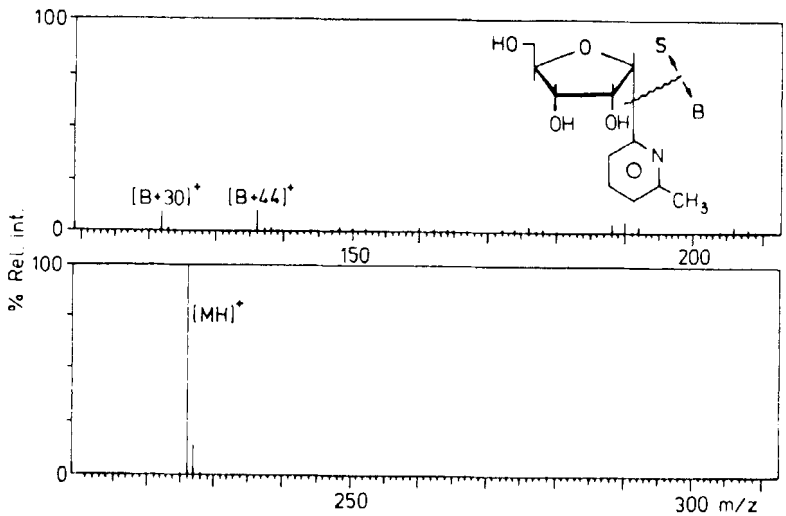


FIG. 2 : Mass spectrum of 2-(α-D-ribofuranosyl)-6-methylpyridine (RT = 7.15 min).

TABLE 1 : DLI/LC-MS mass spectra of pyridine-C-nucleosides I to XI. T_B = 190°C, T_{DC} = 210°C. Relative intensities are given in parenthesis.

Compound	$[MH]^+$	$[B+30]^+$	$[B+44]^+$	Retention time (min)
α-I	212(100)	108(4)	122(5)	4.30
β-I	212(100)	108(2)	122(2)	6.30
α-II	226(100)	122(4)	136(4)	5.29
β-II	226(100)	122(4)	136(2)	14.29
α-III	226(100)	122(8)	136(11)	9.38
β-III	226(100)	122(9)	136(5)	15.34
α-IV	226(100)	122(4)	136(5)	8.53
β-IV	226(100)	122(4)	136(2)	13.18
α-V	226(100)	122(11)	136(12)	7.15
β-V	226(100)	122(8)	136(8)	12.33
α-VI	246(100)*	142(29)*	156(41)*	9.58
β-VI	246(100)*	142(21)*	156(42)*	11.37
α-VII	228(100)	124(35)	138(59)	2.50
β-VII	228(100)	124(17)	138(21)	3.23
VIII•	242(100)	138(14)	152(19)	3.41
α-IX	256(100)	152(2)	166(2)	4.14
β-IX	256(100)	152(3)	166(4)	5.58
α-X	270(100)	166(12)	180(41)	5.53
β-X	270(100)	166(8)	180(10)	7.90
α-XI	284(100)	180(14)	194(23)	9.59
β-XI	284(100)	180(10)	194(12)	12.50

•α,β-anomers not resolved.

* relative intensity for ^{35}Cl.

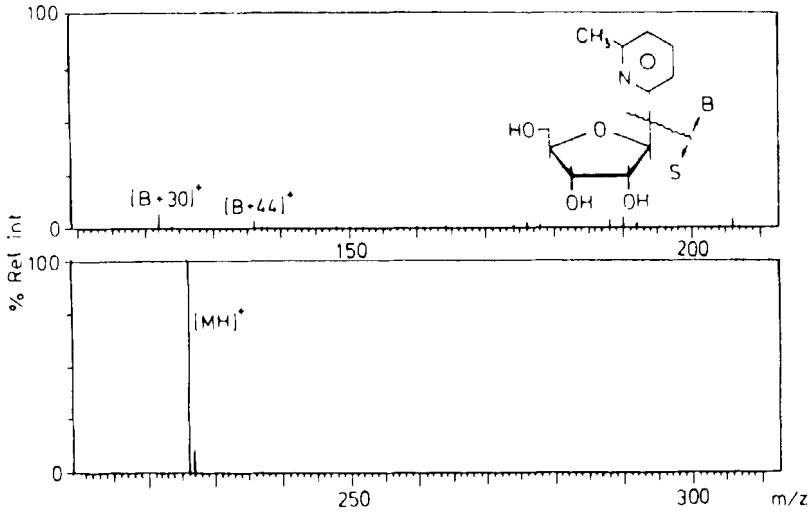


FIG. 3 : Mass spectrum of 2-(β-D-ribofuranosyl)-6-methylpyridine (RT = 12.33 min).

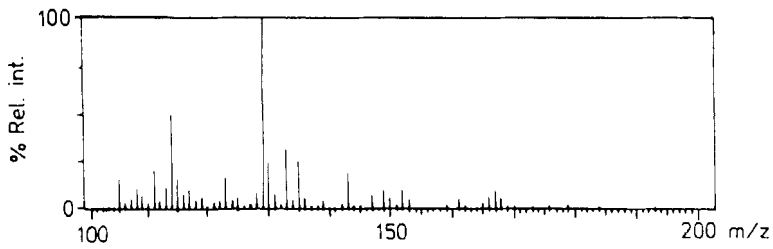


FIG. 4 : Mass spectrum of methanesulphonic acid (RT = 4.44 min ; m/z = 129 corresponds to $[(CH_3SO_3H)(CH_3OH)(H)]^+$-cluster.

TABLE 2 : Comparison of ^{13}C-NMR and LC-MS peak integration data.

	LC-MS		^{13}C-NMR	
	%α	%β	%α	%β
I	38	62	42	58
II	46	54	42	58
III	44	56	46	54
IV	44	56	44	56
V	38	62	48	52

impurities were eluted at, 4.44 min and 10.37 min respectively (FIG. 1).

The mass spectrum of the compound eluting at 4.44 min is depicted in FIG. 4. The ion at m/z = 129 is clearly due to a $[(CH_3SO_3H)(CH_3OH)]H^+$-cluster originating from methane sulfonic acid in the mixture. This impurity is liberated during the cyclisation process of the D-allo- and D-altro-mesylate precursors and is therefore present in all the crude reaction mixtures of C-nucleosides I to XI.

The other compound (RT = 10.37 min), characterized by a protonated molecular ion at $[MH]^+$ = 110 was identified as 6-methyl-2-pyridone. This compound is probably generated during the synthesis of the 2-bromo-methylsubstituted pyridines from the corresponding 2-amino precursors via a Craig diazotation procedure. During such a procedure, 2-pyridones can emerge as by-products[8].

The analysis of 4-(D-ribofuranosyl)-3-chloropyridine (VI) using the same chromatographic conditions, also resulted in the separation of the α- and β-anomers, eluting at 9.58 and 11.37 min respectively (FIG. 6). The corresponding mass spectra are given in TABLE 1.

These compounds were characterized by a protonated molecular ion $[MH]^+$ at m/z = 246 (^{35}Cl) and $[B+30]^+$-(m/z = 142, ^{35}Cl) and $[B+44]^+$-ions (m/z = 156, ^{35}Cl). Also fragment ions at m/z = 218 (^{35}Cl) and 210 (^{35}Cl) were detected. These ions can be explained by the elimination of one and two molecules of H_2O from $[MH]^+$ respectively (FIG. 7).

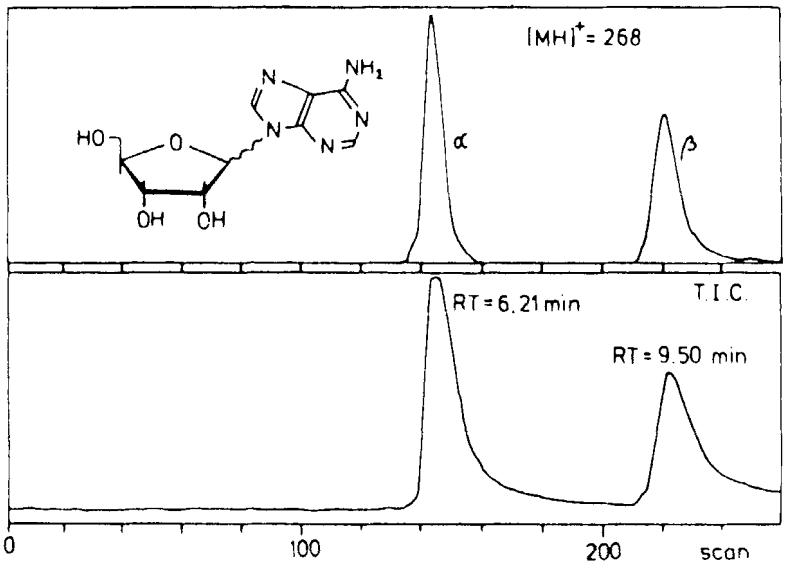


FIG. 5 : Microbore DLI/LC-MS of a mixture of α- and β-adenosine on a 10RP8 column (25 cm x 1 mm I.D.). Eluant : 85% 0.01 M $HCOONH_4$/15% CH_3OH. Flow-rate : 80 µL/min. $T_{desolvation\ chamber}$ = 210°C ; T_{source} = 190°C.

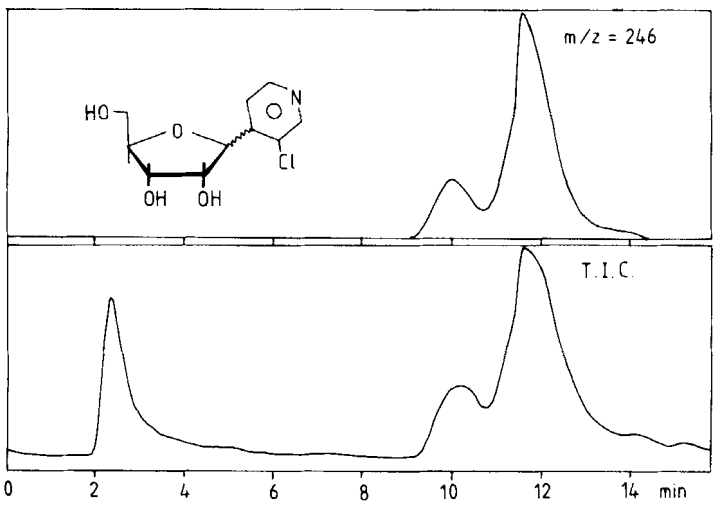


FIG. 6 : Microbore DLI/LC-MS of a crude mixture of 4-(D-ribofuranosyl)-3-chloropyridine (VI). Eluant : 85% 0.01 M $HCOONH_4$/15% CH_3OH ; flow-rate : 80 µL/min. Column : microbore 10RP8 (25 cm x 1 mm I.D.). T_{source} = 190°C ; $T_{desolvation\ chamber}$ = 210°C.

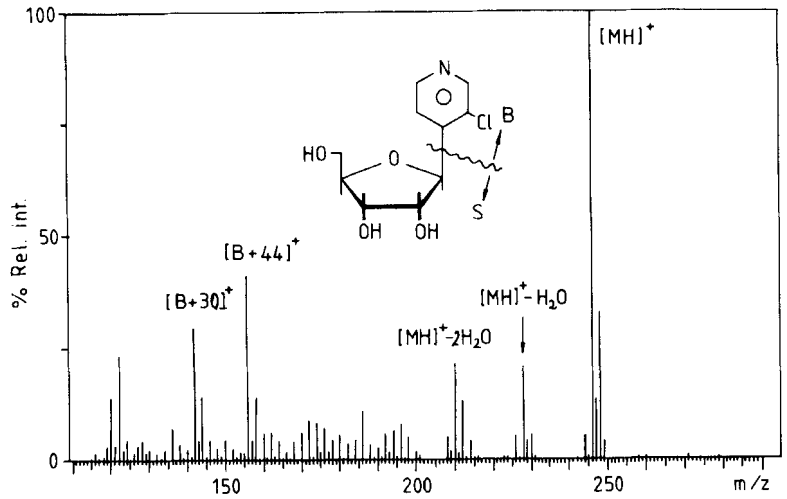


FIG. 7 : Mass spectrum of 4-(α-D-ribofuranosyl)-4-chloropyridine (RT = 9.58 min).

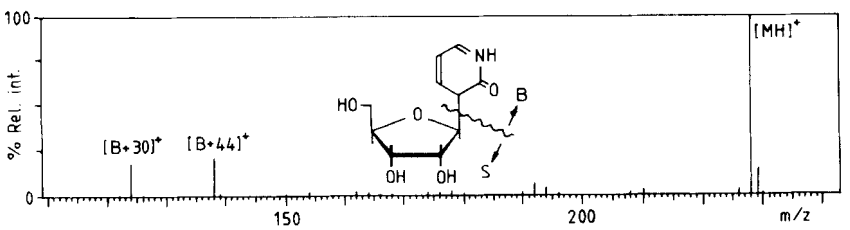


FIG. 8 : Mass spectrum of 3-(β-D-ribofuranosyl)-2-pyridone.

The DLI/LC-MS investigation of a reaction mixture involving the synthesis of 2-fluoro-3-(D-ribofuranosyl)-pyridine were quite interesting and illustrate the power of LC-MS.

The mass spectra of the compounds eluting at 2.50 and 3.23 min respectively, were characterized by m/z-values of 228 ($[MH]^+$), 124 ($[B+30]^+$) and 138 ($[B+44]^+$) (Fig. 8). These m/z-values differ by 2 amu from the values expected for the fluoro-compound. This would indicate that a substitution of the 2-fluoro-atom by a hydroxyl function had resulted in the formation of 3-(D-ribofuranosyl)-2-pyridone (VII). This reaction occurs during the last step of the general synthetic procedure, i.e. the cyclisation step in 1 N HCl. Later on, this substitution reaction was proven by the observation of carbonyl frequencies in the ^{13}C-NMR spectrum at 160.11 and 158.3 ppm respectively.

The series of 2-(D-ribofuranosyl)-3-alkoxypyridines (VII to XI) showed a somewhat different chromatographic behaviour. The appropriate eluant composition was 60% 0.01 M $HCOONH_4$/40% CH_3OH at a flow-rate of 80 µL/min (TABLE 1).

Under these conditions, the α- and the β-anomer of 3-methoxy-2-(D-ribofuranosyl)pyridine (VIII) were not resolved. A DLI/LC-MS run of XI is shown in FIG. 9.

Again in these compounds the base peak was the protonated molecular ion $[MH]^+$, accompanied again by $[B+30]^+$- and $[B+44]^+$-ions. Together with the C-nucleosides, a small amount of methanesulfonic acid was detected.

From these DLI/LC-MS experiments, a strategy was deduced for the semi-preparative clean-up of the C-nucleosides prior to their biological evaluation. Since all impurities mentioned above were quite different in structure from the C-nucleosides I to IX, a purification method was used based upon affinity chromatography[4,6,9,10] followed by semi-preparative reverse phase chromatography using appropriate mixtures of 0.01 M $HCOONH_4/CH_3OH$[7]. The latter technique has allowed the biological evaluation of anomerically pure samples.

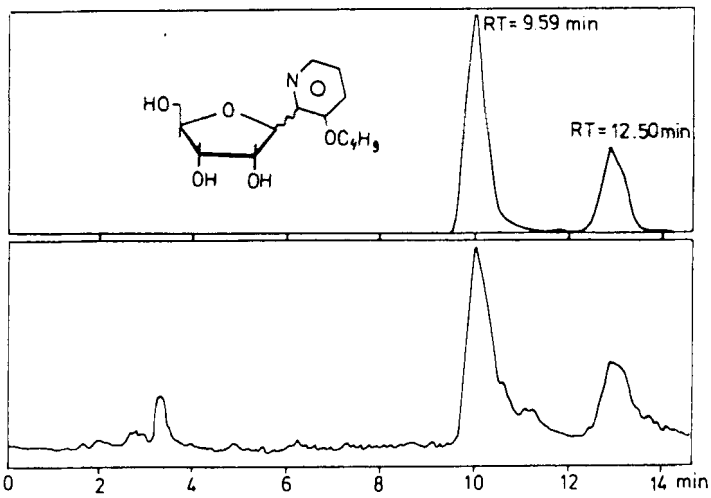


FIG. 9 : DLI/LC-MS analysis of 3-butoxy-2-(D-ribofuranosyl)pyridine (XI). Eluant : 60% 1 M $HCOONH_4$/40% CH_3OH ; flow-rate : 80 µL/min ; column : Microbore 10RP8 (25 cm x 1 mm I.D.). T_{source} : 190°C, $T_{desolvation\ chamber}$: 210°C.

III. Experimental.

1. Products : CH_3OH (HPLC-grade) was purchased from Burdick and Jackson, $HCOONH_4$ from Janssen Chimica and α- and β-adenosine from Sigma.

2. LC-MS :

Aliquots of 1.5 µg were introduced on a microbore 10 RP8 column (25 cm x 1 mm I.D.) (1 µL internal loop). The flow rate was kept constant at 80 µL/min during all experiments. The DLI interface was cooled down to -6°C--8°C with gaseous CO_2 and was coupled to a Riber 10-10B quadrupole mass spectrometer equipped with a SIDAR data system. During the liquid chromatographic/mass spectrometric experiments an ionization chamber and an ion source envelope pressure of respectively 0.6-0.8 mm Hg and $5x10^{-4}$ mm Hg was measured. The ionization energy was kept at 70 eV and the repeller voltage was 0 V. Primary ionization of the solvent was carried out with an emission current of 0.08 mA. The source temperature was 190°C and the temperature of the desolvation chamber was 210°C.

Acknowledgements.

The work is supported by NATO-grant 824/84.

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Received January 19, 1987.