# 5-METHOXYURIDINE, A NEW MODIFIED CONSTITUENT IN tRNAs OF BACILLACEAE*

M. ALBANI, W. SCHMIDT and H. KERSTEN
*Institut für Physiologische Chemie der Universität Erlangen-Nürnberg, 8520-Erlangen, Wasserturmstrasse 5, FGR*

and

K. GEIBEL
*Institut für Organische Chemie der Universität Erlangen-Nürnberg*

and

I. LÜDERWALD
*Institut für Organische Chemie der Universität Mainz, Mainz, FGR*

Received 13 September 1976

## 1. Introduction

In previous communications we have described the occurrence of an unidentified pyrimidine derivative in tRNA of *Bacillus subtilis* [1,2]. The modification of the nucleoside is derived from methionine and concerns the base moiety. The relative frequency of this modified base in tRNA of *B. subtilis* was found to depend on the physiological state of the bacteria. Therefore the unknown constituent was designated as 'P'. The modified base P was suggested to be identical with the unidentified pyrimidine derivative observed by Agris et al. [3] in *Bacillus stearothermophilus*.

In this paper we show that the base P is a common constituent of tRNAs from three bacillus species. A procedure for the purification and isolation of P from tRNA of *Bacillus subtilis* is presented. The identity of the base P with 5-methoxyuridine is proposed on the basis of chromatographic behaviour, ultraviolet absorption, NMR spectra and mass spectrometry.

*The results of this report were presented as a poster at the Xth International Congress of Biochemistry, July 1976, at Hamburg, FGR.

## 2. Materials and methods

Chemicals were from the following sources: L-[*methyl*-$^{14}$C]-methionine (51.2 mCi/mmol); Radiochemical Centre Amersham England. RNAase $T_2$; Calbiochem, USA. Alkaline phosphatase; Worthington, USA. Anion and cation exchange resins AG 1 X 8 and Dowex 50 W X 8; Bio-Rad Laboratories, USA. Cellulose thin layer plates; E. Merck AG; Schleicher & Schüll, West Germany. X-ray films, Osray $T_4$, for autoradiography; Agfa, West Germany. 2-Thio-5-(*N*-methylaminomethyl) uridine, 2-thio-5-carboxymethyluridine methyl ester, were kind gifts from Schering AG, West Berlin and 5-hydroxymethyluridine from Dr Gross, München. 3-Methyluridine and 5-methyluridine; Cyclo Chemical USA. 6-Methyluridine; Sigma USA. All other reagents and solvents: E. Merck AG, West Germany.

*B. subtilis* W 23 was grown in 20 ml minimal medium as described previously [4] under vigorous aeration.

For the preparation of labelled marker a 10 ml overnight culture was diluted into 500 ml fresh medium and grown up to an optical density of

$A_{578}$ = 0.5. Then 8.75 μmol L-[*methyl*-$^{14}$C]-methionine (250 μCi) were added. The cells were grown up to the early stationary phase and then harvested by centrifugation.

For analysis of labelled nucleotides, *Bacillus subtilis*, *Bacillus brevis*, and *Bacillus coagulans* (Deutsche Sammlung für Mikroorganismen, München) were grown up to the stationary phase in 100 ml of glucose–salt medium containing 3.5 μmol of L-[*methyl*-$^{14}$C]-methionine (100 μCi).

Bulk tRNA was isolated by the following procedure: Small amounts of $^{14}$C-labelled bacteria were disrupted before treatment with phenol as described previously [4]. Larger amounts (up to 100 g) of cells were extracted by direct treatment with phenol [5]. The nucleic acids were precipitated at −20°C with ethanol containing 2% potassium acetate. Ribosomal RNA was removed by precipitation with a LiCl-buffer [6]. DNA was separated from RNA by fractionated precipitation with isopropanol in 0.3 M sodium acetate, pH 7.0 [7].

Nucleotides were obtained from labelled tRNAs by digestion with RNAase $T_2$ [8].

The marker, containing unlabeled Up and labelled modified Pp, was isolated after chromatography on cellulose thin-layer plates with isopropanol/HCl/water, 70:15:15 v/v/v. From 2 g bacteria about 40 $A_{260}$ units tRNA, corresponding to 1 × $10^6$ cpm, were obtained.

Nucleotide analysis was performed by two-dimensional thin-layer chromatography [9]. Radioactive spots were detected by autoradiography, eluted with water and counted in dioxane-scintillator.

The nucleoside-marker was isolated after treatment of the nucleotide-marker with alkaline phosphatase [10]. The marker is desalted and deproteinized during the isolation procedure together with unlabeled P-nucleoside.

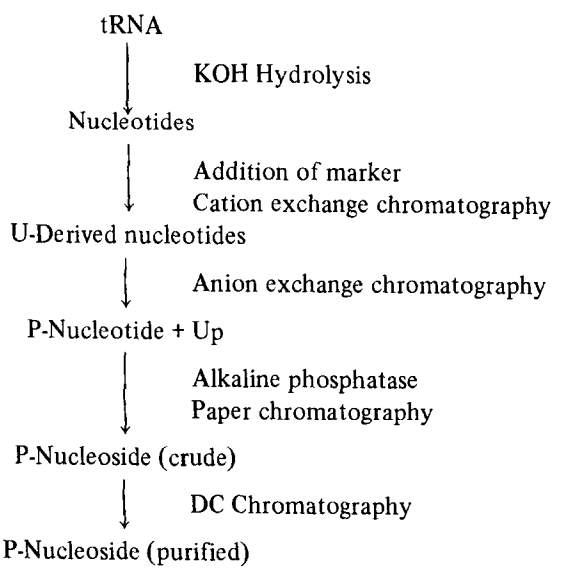
## 3. Results

### 3.1. *Occurrence and relative amounts of P in tRNAs of gram-positive microorganisms*

*Bacillus brevis*, *Bacillus coagulans* and *Bacillus subtilis* were grown in the presence of labelled methionine. The tRNAs were isolated, digested to nucleotides and analyzed. The patterns of labeled nucleotides were found to be almost identical for tRNAs from the three bacterial strains. 5-Methyluridylic acid ($m^5$Up) is not labelled, because the methyl group of $m^5$U in tRNAs of most gram-positive microorganisms is derived from serine and transferred to tRNA by a tetrahydrofolate derivative [11–13]. The methionine derived modified nucleotides and their relative amounts in the three tRNAs are summarized in table 1. Referred to the total amount of methionine-derived nucleotides, set as 100%, $m^7$Gp constitutes about 30%, $m^1$Ap 15% and the new component Pp 15%. Thus P and 1-methyladenine are abundant and characteristic base modifications in tRNA of gram-positive bacteria.

### 3.2. *Isolation procedure of P*

The P nucleoside was isolated from a total amount of 5000 $A_{260}$ units of bulk tRNA of Bacillus subtilis (100 g wet weight). The particular steps of the isolation procedure are shown in the following scheme




The tRNA from *B. subtilis* was hydrolyzed to nucleotides by incubation at 37°C with 10 ml 0.3 M KOH for 16 h. The hydrolysate was acidified with HCl to pH 1.6 and directly applied in portions each corresponding 1000 $A_{260}$ units of tRNA to a column 0.8 × 20 cm of Dowex 50, ($H^+$-form), together with an appropriate amount of labeled marker (see Materials and methods). The large capacity of the

Table 1
Relative distribution of [$^{14}$C-*methyl*]-labelled nucleotides of tRNA from *Bacillus subtilis, Bacillus brevis* and *Bacillus coagulans*

| Compound | Percentage of total radioactivity recovered | | |
|---|---|---|---|
| | *B. subtilis* | *B. brevis* | *B. coagulans* |
| Pp | 15.3 | 15.8 | 16.3 |
| $m^7Gp$ | 27.3 | 30.0 | 32.4 |
| $m^1Gp$ | 8.4 | 8.5 | 8.3 |
| $m^2Gp$ | 5.4 | 4.3 | 4.7 |
| $m^1Ap$ | 14.2 | 15.7 | 17.9 |
| $m^2Ap$ | 7.2 | 8.6 | 6.6 |
| $m^6Ap$ | 6.5 | 3.9 | 3.2 |
| $ms^2i^6Ap$ | 2.9 | 2.9 | 2.7 |
| NmpNp[a] | 12.7 | 10.2 | 10.0 |

[a] Ribose methylated dinucleotides.

column allows chromatography without desalting of the sample. Up and Up-derivatives were eluted with about 50–60 ml of 0.05 N HCl. The other nucleotides remain bound to the column and can be eluted with water [14,15]. Those fractions containing the radioactive marker were evaporated to dryness, dissolved in 2–3 ml of water and adjusted to pH 8.0. Pooled nucleotide fractions corresponding to 5000 $A_{260}$ units of tRNA were further purified on an anion exchange column 0.62 X 40 cm of AG 1 X 8 (formate form). The elution was performed stepwise with gradients shown in fig.1. The major amount of Up and Up-derivatives is eluted with 3 N formic acid. As indicated by the radioactive labelled marker, Pp is slightly retarded with respect to Up (fig. 1). Up was identified by ultraviolet absorption spectrum.

The labeled fractions were pooled, evaporated to dryness and digested to nucleosides by alkaline phosphatase. The digest was applied in portions of about 25 $A_{260}$ units directly on Whatman 3 MM paper sheets and chromatographed descending with isopropanol/water/ammoniumhydroxide, 7:2:1 v/v/v. During this procedure P is desalted and separated further from other U-nucleosides. The labelled spots were detected by autoradiography and eluted two times with 1 ml of water. The final purification steps were performed on thin-layer plates with the same solvent and repreated until uridine was completely separated from P. The $R_X$-value of the P-nucleoside relative to U, is 0.82.

The final samples used for NMR spectroscopy and mass spectrometry were further purified on thin-layer plates that were washed two times with the solvent system in two dimensions.

### 3.3. *Identification of P*

The ultraviolet spectra of the purified nucleoside P in acidic, neutral and basic solution (fig.2) are similar to the ultraviolet spectra of 5-methyluridine

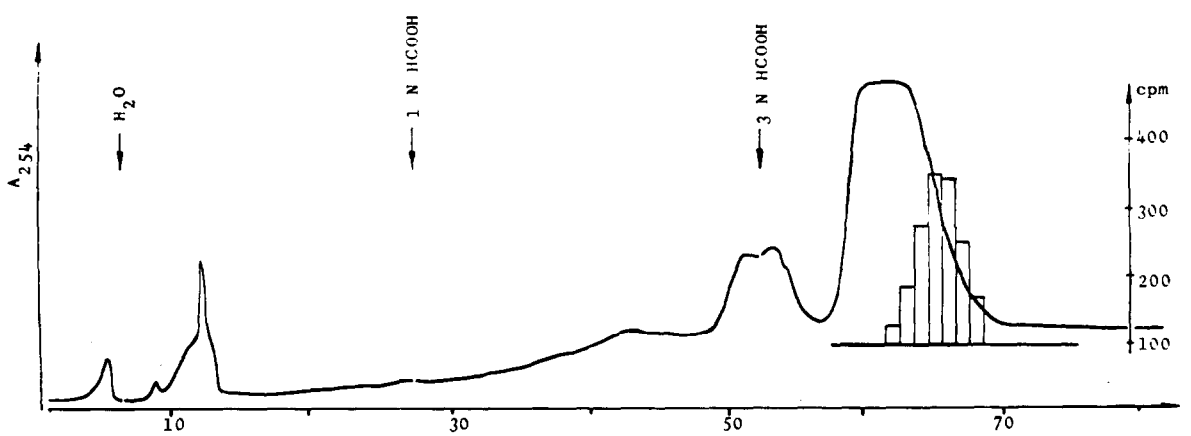



Fig.1. Enrichment of P nucleotide by anion exchange chromatography of Up-derivatives. 1000 $A_{260}$ nucleotides (dissolved and adjusted to pH 8) were applied to a column (0.62 X 40 cm) AG 1 X 8, (formate form). Elution (32 ml/h) was carried out with following gradients: (a) $H_2O \rightarrow$ 1 N HCOOH; (b) 1 N HCOOH $\rightarrow$ 3 N HCOOH; (c) 3 N HCOOH. Fractions of 8 ml were collected. The P nucleotides were eluted with 3 N HCOOH as indicated by the radioactive 'marker' (see columns).

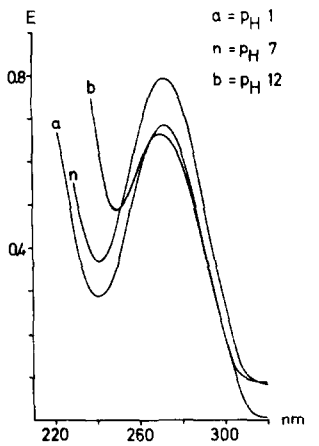



Fig.2. Ultraviolet absorption spectra of the nucleoside P at different pH values: a ≈ 0.1 N HCl, n = $H_2O$, b = 0.1 N ammoniumhydroxide.

and 5-carboxymethyluridine [16]. The characteristics of the spectra of the three compounds are: (i) the bathochromic shift relative to uridine, (ii) the identity of the spectra, at pH 1.0 and at pH 7.0, and the shift of the extinction minimum, at pH 12, to higher wavelength, (iii) a decrease in the absorption, at pH 12, at the maximum and a concomitant increase in the minimum.

The radioactive labelled marker containing uridine and P-nucleoside was cochromatographed on thin-layer plates with available modified nucleosides. In the solvent system isopropanol/HCl/water, 70:15:15 v/v/v the nucleotides and nucleosides of U and P are not separated, but differ in $R_F$-values from other nucleosides except 2-thio-5-carboxymethyluridine (table 2). The latter compound has not yet been found in nucleic acids. In the solvent system isopropanol/$H_2O$/ammoniumhydroxide, 7:2:1, v/v/v, P is separated from uridine and 2-thio-5-carboxymethyluridine. The $R_F$-value of P in the acidic solvent system is different from the $R_F$-values of 3-methyluridine, 5-methyluridine, 6-methyluridine, 2-thio-5-carboxymethyluridine methyl ester, and 2-thio-5-(*N*-methylaminomethyl)uridine and 5-hydroxymethyluridine (table 2).

PMR spectroscopy was carried out with a JEOL INM-PS 100-PFT instrument equipped with a 20 K computer. The sample (40–50 μg) was dissolved in 200 μl 99.9% $D_2O$. The spectrum is shown in fig.3.

Table 2
Methylated uridine derivatives differing from the P-nucleoside in chromatographic behaviour

| Compound | $R_X$-value | |
|---|---|---|
| | 1[a] | 2[b] |
| 3-Methyluridine | 1.25 | 1.63 |
| 5-Methyluridine | 1.20 | 1.20 |
| 6-Methyluridine | 1.20 | – |
| 2-Thio-5-carboxymethyluridine methyl ester | 1.41 | – |
| 2-Thio-5-carboxymethyluridine | 1.00 | 0.25 |
| 2-Thio-5-(*N*-methylaminomethyl)-uridine | 0.46 | – |
| 5-Hydroxymethyluridine | 0.83 | – |

$R_X$-value = $R_F$-value of the examined compound divided by the $R_F$-value of uridine.

[a] 1 solvent system: isopropanol/HCl/$H_2O$, 70:15:15, v/v/v.
[b] 2 solvent system: isopropanol/ammoniumhydroxide/$H_2O$, 7:1:2, v/v/v.

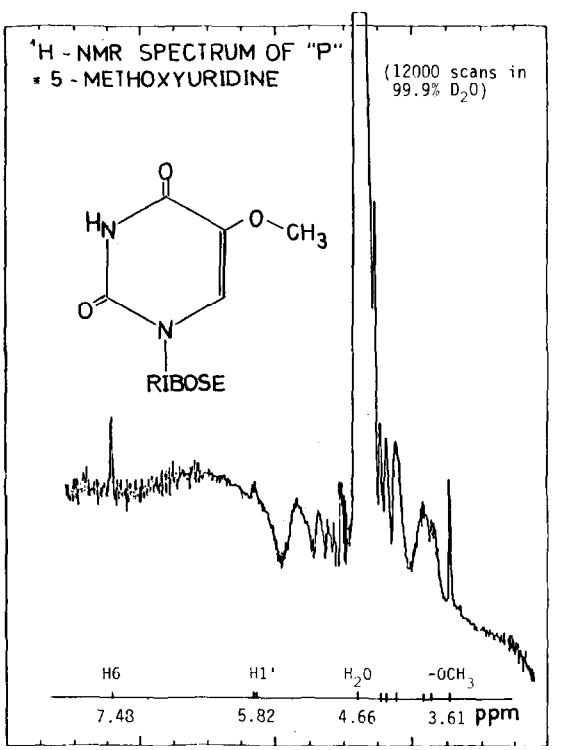



Fig.3. $^1H$ NMR spectrum of P at 100 MHz, 22°C. The nucleoside (40 μg–50 μg) was dissolved in 99.9% $D_2O$. The spectrum was obtained by a JEOL INM-PS 100 PFT instrument with a 20 K computer after 12 000 scans. δ-values, relative to $H_2O$, respectively HOD δ = 4.66 ppm.

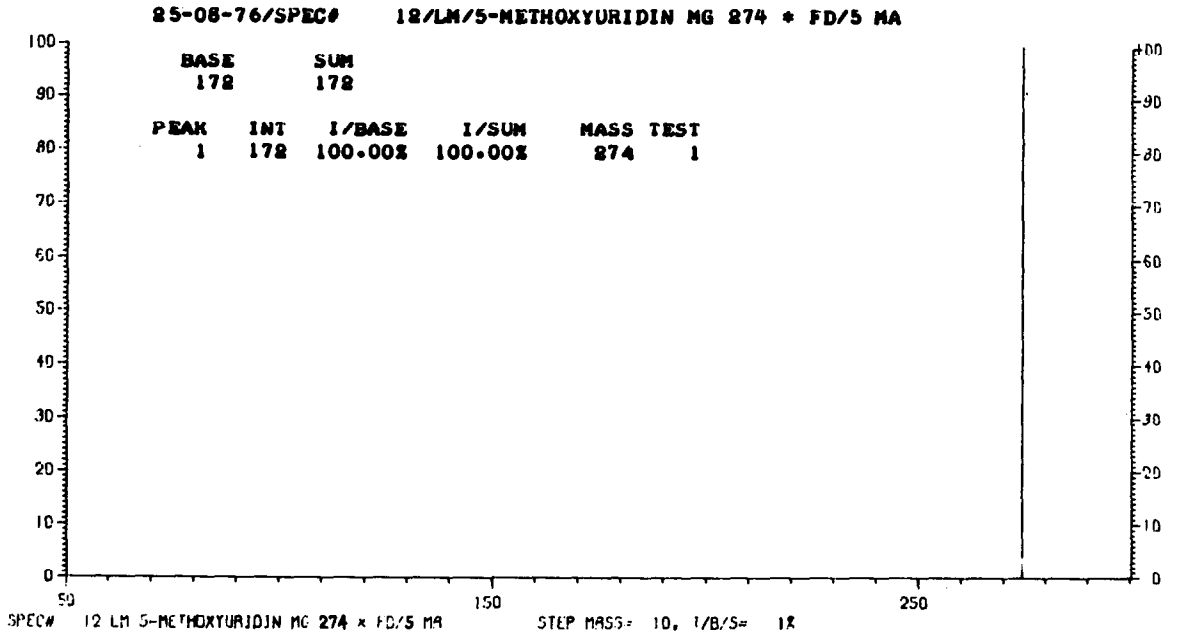



Fig.4. Mass spectrum of the nucleoside P. The mass is identical with 5-methoxyuridine, mol. wt 274.

Chemical shift values are relative to $H_2O$ respectively HOD = 4.66 ppm. Compared with the PMR spectrum of uridine the signal of the proton in position 5 ($\delta$ = 5.77 ppm) is missing and that for H 6 (uridine $\delta$ = 7.75 ppm) now shows a singlet at 7.48 ppm. For uridine this signal is a doublet derived from coupling with the proton in position 5. A new signal is obtained at 3.61 ppm which can be interpreted to originate from three protons of an $-OCH_3$ group.

Mass spectrometry was carried out with a double-focussing mass spectrometer Varian MAT 711. The compound was ionized by field desorption. The spectrum which was monitored by a 12 K computer Varian 6201, revealed a single molecule peak, corresponding to a mass of 274 (fig.4).

## 4. Conclusion

The new modified uracil derivative P occurs in tRNAs from three gram-positive bacillaceae. From the experimental data obtained with tRNA from *B. subtilis* we conclude that the new constituent is identical with 5-methoxyuridine. This conclusion is based on the following facts and considerations.

The ultraviolet absorption spectra at different pH values show that the compound in question is an uracil-derivative.

From the chromatographic behaviour of the modified nucleoside in polar solvent systems it is evident that P is more polar than uridine. P cannot be a plain methylated derivative of uridine because a plain methylation renders a molecule less polar. Therefore the higher polarity of P, compared to U, is assumed to result from an additional oxygen.

The PMR spectrum shows substitution of the proton at position 5 of the molecule and exhibits a new signal which is interpreted as signal from $-OCH_3$.

Mass spectrometry reveals a molecular weight of 274. 5-Methyluracilriboside has a molecular weight of 258. Thus, in addition to the methyl group, an atom or group must be present with a molecular weight of 16. This result together with the other data prove the presence of one oxygen atom. Since P is not identical with 5-hydroxymethyl-uridine (table 2) we conclude that P is identical with 5-methoxyuridine, a new constitutent of tRNA.

The new modified base of *B. subtilis* tRNA may

be closely related to the V-base, uracil-5-oxy acetic acid, present in $tRNA_1^{Ser}$ and $tRNA^{Val}$ of *E. coli* [17,18]. Both modifications can be considered chemically as substitution products of 5-hydroxy-uracil.

Uracil-5-oxy acetic acid is present in tRNAs of *E. coli* in the anticodon loop at the wobble position [17,18]. 5-Methoxyuridine occurs in $tRNA^{Thr}$, $tRNA^{Val}$ and $tRNA^{Ala}$ of *B. subtilis* at the same position (personal communication from K. Murao, T. Hasegawa and H. Ishikura, Japan).

We have shown that submodified tRNA from *B. subtilis* accepts methyl groups from SAM in vitro with the 5-methoxyuracil-specific tRNA methyltransferase of *B. subtilis* [2]. Submodified, but not mature tRNA, from *E. coli* can also serve as a substrate for this specific enzyme (unpublished result, Arnold, Kersten). We therefore suggest, that the 5-methoxyuracil-specific tRNA methyltransferase from *B. subtilis* recognizes a precursor that is common in *B. subtilis* and *E. coli* tRNAs. The precursor is suggested to be 5-hydroxyuracil. The biosynthetic pathway of 5-methoxyuridine in tRNA of gram-positive microorganisms is currently being investigated.

## Acknowledgements

The authors thank Professor Dr H.-J. Bestmann, Erlangen, for cooperation. We are indebted to the 'Deutsche Forschungsgemeinschaft' (grant Ke 98/11) and the 'Fond der Chemischen Industrie' for support of this work.

## References

[1] Kersten, H. and Kersten, W. (1975) in FEBS, Proc. 9th Meeting, Budapest 1974, Vol. 34, Post Synthetic Modification of Macromolecules, pp. 99–110, North-Holland, Amsterdam.
[2] Arnold, H. H., Schmidt, W., Raettig, R., Sandig, L., Domdey, H. and Kersten, H. (1976) Arch. of Biochem. and Biophys. in press.
[3] Agris, P. F., Koh, H. and Söll, D. (1973) Arch. of Biochem. and Biophys. 154, 277–282.
[4] Arnold, H. H. and Kersten, H. (1973) FEBS Lett. 36, 34–38.
[5] Doi, R. H. (1971) in: Protein Biosynthesis in Bacterial Systems, (Last, J. A. and Laskin, A. J., eds) pp. 68–88, Maral Dekker, New York.
[6] Avital, S. and Elson, D. (1969) Biochim. Biophys. Acta 179, 297–307.
[7] Zubay, G. (1962) J. Mol. Biol. 4, 347–356.
[8] Brownlee, G. G. (1972) in: Determ. of Sequences in RNA, p. 254, American Elsevier North-Holland ICN, New York.
[9] Nishimura, S., Harada, F., Narashima, U. and Seno, T. (1967) Biochim. Biophys. Acta 142, 133–148.
[10] Randerath, E., Yu, C.-T. and Randerath, K. (1972) Anal. Biochem. 48, 172–198.
[11] Arnold, H. H., Schmidt, W. and Kersten, H. (1975) FEBS Lett. 52, 62–65.
[12] Kersten, H., Sandig, L. and Arnold, H. H. (1975) FEBS Lett. 55, 57–60.
[13] Schmidt, W., Arnold, H. H. and Kersten, H. (1975) Hoppe Seyler's Zeitschr. Physiol. Chem. 356, 275.
[14] Katz, S. and Comb, D. G. (1963) J. Biol. Chem. 238, 3065–3067.
[15] Runeckles, V. C. and Das, G. (1975) Chrom. 112, 240–243.
[16] Hall, R. M. (1971) in: The Modified Nucleosides in Nucleic Acids, Columbia University Press, New York.
[17] Ishikura, H., Yamada, Y. and Nishimura, S. (1971) FEBS Lett. 16, 68–70.
[18] Harada, F., Kimura, F. and Nishimura, S. (1969) Biochim. Biophys. Acta 182, 590–592.