32. Biosynthetic Production of [N^2,1,3,7,9-^{15}N]Guanosine and [1,3,7,9-^{15}N]Inosine and Conversion into [N^6,1,3,7,9-^{15}N]Adenosine for Structure Elucidation of RNA by Heteronuclear NMR

by **Annette C. Niemann**[1]), **Mónica Meyer**[1]), **Thomas Engeloch**[1]), **Oliver Botta**[1]), **Alfons Hädener**, and **Peter Strazewski***

Institut für Organische Chemie der Universität Basel, St. Johanns-Ring 19, CH–4056 Basel

(7.XI.94)

A procedure was developed for the biosynthetic preparation of ^{15}N-labelled guanosine and inosine through the action of a mutant *Bacillus subtilis* strain. Crude [N^2,1,3,7,9-^{15}N]guanosine and [1,3,7,9-^{15}N]inosine were isolated from the culture filtrate by precipitation and anion-exchange chromatography (*Scheme 1*). No cell lysis and no enzymatic degradation was necessary. The per-isobutyrylated derivatives **1** and **2** were isolated from a complex mixture, purified by virtue of their different lipophilicity, and separated in three steps involving normal- and reversed-phase silica-gel chromatography. One litre of complex nutrient medium yielded 8.44 mmol of guanosine derivative and 2.84 mmol of inosine derivative with high average ^{15}N enrichment (83.5 and 91.9 atom-%, resp.). [N^6,1,3,7,9-^{15}N]Adenosine (**4**) was obtained from 2',3',5'-tri-*O*-isobutyryl[1,3,7,9-^{15}N]inosine (**1**) through the ammonolysis of its 1,2,4-triazolyl derivative with aqueous ^{15}NH$_3$ (*Scheme 2*).

Introduction. – The advent of multidimensional heteronuclear ^{1}H-NMR spectroscopy for the elucidation of the solution structure of large biomolecules [1] [2] led to a number of investigations aiming at the preparation of defined ^{15}N-labelled RNA fragments [3] [4]. The general approach was a biosynthetic one: Microorganisms grown on minimal media containing [^{15}N]ammonium salts were used to produce labelled RNA. After enzymatic degradation to the nucleosides and enzymic phosporylation, all four ^{15}N-labelled ribonucleoside 5'-triphosphates were obtained in 1–100 µmol amounts per litre of culture medium. Those were used as substrates for an enzymic *in vitro* RNA synthesis by T7 RNA polymerase.

Our approach divides the preparation of ^{15}N-labelled ribonucleosides into a biosynthetic and a synthetic one, with the aim to obtain g-quantities of the nucleosides for their use in both *in vitro* and chemical [U-^{15}N]RNA synthesis. Here, we describe the preparation of the purine nucleosides [1,3,7,9-^{15}N]inosine and [N^2,1,3,7,9-^{15}N]guanosine. They were directly obtained from a conventionally cultivated mutant *Bacillus subtilis* strain NA6601 [5]. Under certain growth conditions, these *Gram*-positive bacteria are capable of releasing large quantities of guanosine (G) and inosine (I) into the growth medium. This greatly simplifies the isolation and purification procedure. Subsequently, [1,3,7,9-^{15}N]inosine was chemically converted into [N^6,1,3,7,9-^{15}N]adenosine.

Results and Discussion. – *Production, Isolation, and Purification of the Main Metabolites.* Optimal aerobic growth of *B. subtilis* NA6601 required a medium based on sorbitol and autolyzed yeast (SD medium) for precultures and a complex medium based on

---

[1]) Diploma thesis (*A. N.* 1991, *M. M.* and *T. E.* 1992, *O. B.* 1993).

glucose and corn steep liquor (FM medium) for the overproduction of nucleosides [6]. In a typical production, aliquots (1 part) of precultures that had been grown at 37° for 24 h were used to inoculate portions (3 parts; v/v) of FM medium. These cultures were incubated at 37° on a rotary shaker. The production of inosine and guanosine in the FM culture was quantitatively followed in real time by reversed-phase HPLC; the culture broth was analyzed directly. To obtain large quantities of nucleosides in highly enriched ^{15}N-labelled form, it was necessary to optimize the composition of the FM medium with respect to the concentrations of various N sources while a maximum nucleoside production and a high inosine $vs.$ guanosine ratio had to be maintained.

Two N sources that are readily metabolized by the organism, and that can be added economically in the ^{15}N-labelled form, $(NH_4)_2SO_4$ and urea, make up for $ca.$ 80% of the total N content of the FM medium in its original composition. The rest is contributed by glutamate (7.4%), corn steep liquor ($ca.$ 6%), ribonucleic acid (2.7%), and N carried over from the preculture in SD medium (4%). The main C source is D-glucose. Phosphorus originates from three sources, commercially available RNA from $Torula utilis$ (44%), corn steep liquor (11%), and P carried over from the preculture (45%).

To reduce the ^{14}N content in the FM medium, we tested whether monosodium L-glutamate could be omitted or replaced by other ingredients. The biosynthetic precursor of the N(1) atom of inosine and guanosine is aspartate which is in biosynthetic equilibrium with glutamate: glutamate + oxaloacetate $\rightleftharpoons$ aspartate + 2-oxoglutarate [7]. The addition of either L-glutamic or L-aspartic acid to the FM medium was found to be necessary for an effective overproduction. Although glutamate is biosynthesized from 2-oxoglutarate, the replacement of the former compound by the latter was ineffective. But the substitution of monosodium L-glutamate by small amounts of L-aspartic acid ($^1/_{10}$ of the original molar concentration of L-glutamate) was found to be ideal for both a high yield of nucleosides and an optimal inosine production.

In other test experiments, the requirement to include RNA as an ingredient to the FM medium was checked: the nucleosides accumulated to a much lower extent when RNA was either omitted or added at increased concentrations. Various other details of the fermentation protocol, such as the size of cultures, the extent of aeration, the transfer of $inocula$, $etc.$, were also subjected to careful optimization. $Table 1$ shows the contribution of various N sources to the total N content of the optimized nutrient medium during the production of ^{15}N-labelled nucleosides.

Initially and unexpectedly, when unlabelled $(NH_4)_2SO_4$ was replaced by $(^{15}NH_4)_2SO_4$ (from $^{15}NH_4Cl$ and H_2SO_4), the nucleoside production was found to be markedly inhibited. Purification of the ^{15}N-labelled material was, therefore, necessary prior to its use in the fermentations. X-Ray fluorescence of crystalline commercial $^{15}NH_4Cl$ ($Cambridge$

Table 1. $Sources of$ N $in the Nutrient Medium Used for the Overproduction of$ ^{15}N-$Labelled Nucleosides$ by B. subtilis $NA6601$

Component	N [%]	Component	N [%]
$(^{15}NH_4)_2SO_4$	40.5	Corn steep liquor (2.7–4.5% N)[a]	7.1
$(^{15}N_2)$Urea	43.9	RNA from $T. utilis$ (15.6% N)[a]	3.0
L-Aspartic acid[a]	0.85	$Inoculum$ carried over from preculture[a]	4.6

[a])   Unlabelled.

Isotope Laboratories) revealed a severe contamination by a Br-containing compound (0.1–1% Br). High-temperature GC/MS analysis of CH_2Cl_2 extracts of an aqueous $^{15}NH_4Cl$ solution did not reveal any significant organic impurities, suggesting that the Br-containing compound was either inorganic (Br^- could be ruled out by anion chromatography), too thermolabile, or not sufficiently volatile for GC. This impurity was eliminated by H_2O-vapour distillation of $^{15}NH_3$ into H_2SO_4, but the inhibition remained. ($^{15}NH_4)_2SO_4$ was subsequently purified by passing an aqueous solution over a strongly metal-chelating cation-exchange resin (*Chelex 100*, Na^+ form). The metals were washed off the resin distilled 1N HCl. Inductively-coupled-plasma mass spectra partly coupled with optical emission spectroscopy (ICP-MS, ICP-OES) of the HCl solution revealed a 22-fold lower Cu (0.12 ppm) and a 12-fold higher Sr content (7.26 ppm), as compared to a reference HCl solution that was obtained from extracted, distilled, and *Chelex*-purified unlabelled material (2.76 ppm of Cu and 0.6 ppm of Sr, NH_4Cl from *Merck*). No increased radioactivity, possibly owing to ^{90}Sr, was found in $^{15}NH_4Cl$. ICP-MS and ICP-OES analysis of HCl washings derived from *Chelex*-purified ($^{15}N_2$)urea (*CIL*) revealed an approximately two-fold higher Al, B, Ba, Sr, and Zn, and a three-fold higher Pb content (*vs.* urea from *Merck*). All other contaminations by heavy or potentially toxic metals were within the range or even lower in comparison to the reference solutions. Thus, an extraction with CH_2Cl_2, a H_2O-vapor distillation into analytical-grade H_2SO_4 and subsequent *Chelex* purification of ($^{15}NH_4)_2SO_4$ and a *Chelex* purification of ($^{15}N_2$)urea were carried out prior to an optimal nucleoside production.

The entire production of ^{15}N-labelled G and I was carried out starting from 9.32 g of ($^{15}N_2$)urea (99% ^{15}N; 300 mmol of ^{15}N) and 18.92 g of ($^{15}NH_4)_2SO_4$ (99% ^{15}N; 282 mmol of ^{15}N) in three series in which a total of 45 small FM cultures (20–40 ml each, 1020 ml total) were grown. Substantial production of the nucleosides began only after 20 h, followed by an exponential growth phase until, after 78 to 80 h, the amount of I and G began to drop owing to catabolic degradation. The pH rose from 6.8 to 8.1–8.5 during the growth period. After 80 h, the culture broths were combined into two lots. It was possible to store these at −30° without loss of labelled nucleosides. Later, the lots were boiled separately and filtered hot over *Celite*. G crystallized preferentially in the filtrate. After several precipitations, the last mother liquor was set to pH 11, and residual $^{15}NH_3$ was collected by rapid distillation into 2N HCl. Thus, 5.85 g of $^{15}NH_4Cl$ (96 atom-% ^{15}N; 103 mmol of ^{15}N, 18%) could be regenerated. The residual solid was separated on an anion-exchange resin to furnish I-enriched fractions. All I- and G-containing fractions were pooled. The yield at this stage was 2.2 g of I and 7.1 g of G, as shown by HPLC analysis of the culture filtrates.

Subsequently, the crude solids and oils were treated with an excess of isobutyryl chloride in pyridine. A mixture of crude 2′,3′,5′-tri-*O*-isobutyryl[1,3,7,9-^{15}N]inosine (**1**) and N^2-isobutyryl-2′,3′,5′-tri-*O*-isobutyryl[N^2,1,3,7,9-^{15}N]guanosine (**2**) was obtained together with mostly non-nucleoside impurities (TLC). The purification and separation of **1** and **2** was accomplished in three chromatographic steps using both normal-phase and reversed-phase silica gels (*Scheme 1*). First, a flash chromatography (FC) over normal-phase SiO_2 using a step gradient of MeOH in CH_2Cl_2 separated in four runs most of the non-nucleosidic from the nucleosidic compounds. Four pooled FC fractions X, A, B, and C resulted. X contained **2** together with a hitherto unidentified compound, A contained mainly **2**, B a mixture 2/1, and C mainly **1** largely free of **2**. In a second step, each of these

1   **2**
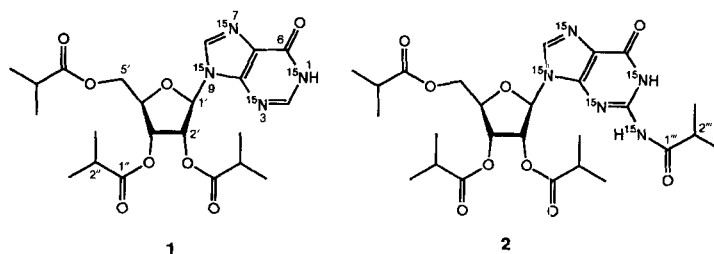
fractions was submitted to preparative UV-detected reversed-phase medium-pressure liquid chromatography (MPLC) using a time-programmed H₂O/MeOH gradient. The gradient was optimized so as to efficiently separate **1** from **2**. Because of their low solubility in the initial eluant (H₂O/MeOH 1:1), the FC fractions were applied as impregnates onto an MPLC precolumn. A number of reversed-phase MPLC runs were needed for the complete separation of all **1** and **2**. According to reversed-phase HPLC analysis, the original MPLC fractions were pooled into two main fractions, the I and the G fraction (4.0 and 5.6 g, resp.). Besides, 12 other reversed-phase MPLC fractions were kept for an elementary and ^{1}H-NMR analysis, to determine whether other useful ^{15}N-containing compounds had been isolated (*e.g.* [1,3,7,9-^{15}N]hypoxanthine). Finally, the I and G fractions were each purified in a last isocratic normal-phase MPLC step resulting in 5.04 g

Scheme 1. *Workup and Purification of the Biosynthetic Production of ^{15}N-Labelled Inosine and Guanosine*
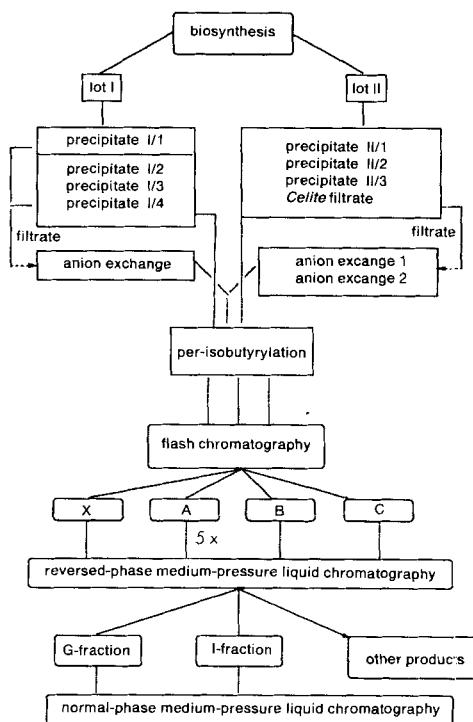
of **2** and 1.44 g of **1** (36 and 37%, resp., with respect to the HPLC-monitored contents in the culture broth). The 'A_{259} purity' of both compounds was determined by HPLC to be 87–95% for **1** and 97–100% for **2**. The actual purities were > 95% as determined by ^{1}H-NMR.

Spectroscopic Characterization. The FAB$^+$-MS of **1** reveals a collection of signals at m/z 479–485. The intensity of m/z 483 is 20% of the base peak (triisobutyrylribose cation at m/z 343). These signals represent a mixture of isotopomers with different degrees of protonation (*Table 2*). The degree of protonation is determined by measuring unlabelled **1** under the same conditions. The same distribution of $[M - H]^+$, M^+, $[M + H]^+$, $[M + 2 H]^+$, and $[M + 3 H]^+$ is carried over to each isotopomer. Seven significant peaks and five degrees of protonation are expressed as a system of 7 equations, 5 constants, and 5 unknowns A to E representing the fraction of each of the 5 possible isotopomers ($^{14}N_4$)- to ($^{15}N_4$)-**1** contained in the mixture. The over-determined equation system is solved by a weighted linear regression analysis according to *Eqn. 1.* $\vec{x}$ is the solution vector, M is the 7×5 matrix expressing the degrees of protonation, $\vec{c}$ contains the mean relative intensities ϕ_i of mass peaks ($i = 1$–7) and G_y is the inverted variance matrix $C_y(\sigma_i)$. The values for ϕ_i and σ_i are taken from *Table 2*; C_y is calculated according to *Eqn. 2.*

$$\vec{x} = \begin{pmatrix} A \\ \cdots \\ E \end{pmatrix} = (M^t \times G_y \times M)^{-1} \times M^t \times G_y \times \vec{c}; \quad \vec{c} = \begin{pmatrix} \phi_1 \\ \cdots \\ \phi_7 \end{pmatrix} \tag{1}$$

$$C_y = \begin{pmatrix} \dfrac{1}{\sigma_1^2} & 0 & \cdots & 0 \\ 0 & \dfrac{1}{\sigma_2^2} & 0 & \cdots \\ \cdots & 0 & \cdots & 0 \\ 0 & \cdots & 0 & \dfrac{1}{\sigma_7^2} \end{pmatrix} = G_y^{-1} \tag{2}$$

Table 2. *Mass, Relative Intensities, and Degree of Protonation of the Molecular Cations of* **1**.
$A = (^{15}N_0, {}^{14}N_4)$, $B = (^{15}N_1, {}^{14}N_3)$, $C = (^{15}N_2, {}^{14}N_2)$, $D = (^{15}N_3, {}^{14}N_1)$, $E = (^{15}N_4, {}^{14}N_0)$.

m/z	Relative intensity ϕ_i [%] [a])	Standard deviation/rel. int. σ_i [a])	$[M - H]^+$ 1.43±0.18 [%] [b])	M^+ 5.70±0.26 [%] [b])	$[M + H]^+$ 71.4±0.6 [%] [b])	$[M + 2H]^+$ 18.6±0.4 [%] [b])	$[M + 3H]^+$ 3.02±0.13 [%] [b])
479	0.35	0.1595	C	B	A		
480	1.04	0.1546	D	C	B	A	
481	4.75	0.0568	E	D	C	B	A
482	20.3	0.0099		E	D	C	B
483	57.2	0.0087			E	D	C
484	14.0	0.0214				E	D
485	2.38	0.0588					E

[a]) From 10 measurements.
[b]) Degree of protonation from FAB$^+$-MS of unlabelled lib$_3^{2',3',5'}$, mean value and standard deviations from 9 measurements.

Thus, biosynthetic inosine is composed of 73.87% of $(^{15}N_4)$-, 21.64% of $(^{15}N_3,^{14}N_1)$-, 3.26% of $(^{15}N_2,^{14}N_2)$-, 0.66% of $(^{15}N_1,^{14}N_3)$-, and 0.37% of $(^{14}N_4)$-isotopomers, resulting in an average ^{15}N content of 91.9%. During the productive phase, apparently no significant catabolism of RNA nucleobases or other exogeneous ^{14}N-containing compounds produced reusable $^{14}NH_4^+$ cations, or else the content of the $(^{14}N_4)$-isotopomer would have been higher. Nevertheless, the quite high content of $(^{15}N_3,^4N_1)$inosine indicates that added unlabelled L-aspartic acid, the direct biosynthetic precursor of the N(1) atom of both metabolites, was efficiently incorporated.

The FAB$^+$-MS of **2** reveals two collections of signals at m/z 221–229 and 563–571. The intensity of m/z 227 and 569 is 23 and 53%, respectively, with respect to the base peak (triisobutyrylribose cation at m/z 343). These signals represent a mixture of the isotopomeric N^2-isobutyrylguanine and molecular ions, respectively, with different degrees of protonation. The same analysis as above (*Eqns. 1* and *2*, except $i = 1$–9) is carried out using the signals from the molecule ions, since their intensities are higher and the standard deviations correspondingly lower. A control using the N^2-isobutyrylguanine intensities shows no significant deviations. The over-determined equation system consists of 9 significant relative peak intensities and 5 degrees of protonation resulting in a system of 9 equations, 5 constants, and 6 unknowns A–F, representing the fraction of the 6 possible isotopomers $(^{14}N_5)$- to $(^{15}N_5)$-**2** in the mixture (*Table 3*).

Table 3. *Mass, Relative Intensities, and Degree of Protonation of the Molecular Cations of 2.*
$A = (^{15}N_0,^{14}N_5)$, $B = (^{15}N_1,^{14}N_4)$, $C = (^{15}N_2,^{14}N_3)$, $D = (^{15}N_3,^{14}N_2)$, $E = (^{15}N_4,^{14}N_1)$, $F = (^{15}N_5,^{14}N_0)$.

m/z	Relative intensity ϕ_i [%] [a]	Standard deviation/rel. int. σ_i [a]	$[M-H]^+$ 1.69±0.06 [%] [b]	M^+ 13.0±0.18 [%] [b]	$[M+H]^-$ 62.6±0.43 [%] [b]	$[M+2H]^+$ 18.8±0.25 [%] [b]	$[M+3H]^+$ 3.85±0.21 [%] [b]
563	0.67	0.0817	B	A			
564	4.66	0.0314	C	B	A		
565	3.13	0.0254	D	C	B	A	
566	1.76	0.0495	E	D	C	B	A
567	7.36	0.0229	F	E	D	C	B
568	25.32	0.0155		F	E	D	C
569	43.24	0.0068			F	E	D
570	11.97	0.0186				F	E
571	1.88	0.0379					F

[a]   From 10 measurements.
[b]   Degree of protonation from FAB$^+$-MS of unlabelled ib^{2}Gib$^{2',3',5'}$, mean value and standard deviation from 9 measurements.

Thus, biosynthetic guanosine is composed of 58.75% of $(^{15}N_5)$-, 26.75% of $(^{14}N_1,^{15}N_4)$-, 4.48% of $(^{14}N_2,^{15}N_3)$-, 0.31% of $(^{14}N_3,^{15}N_2)$-, 2.8% of $(^{14}N_4,^{15}N_1)$-, and 6.91% of $(^{14}N_5)$-isotopomers, resulting in an average ^{15}N content of 83.5%. The relatively high content of unlabelled guanosine indicates that, during the productive phase, exogeneous unlabelled RNA was degraded to the nucleosides by extracellular RNAses. The resulting phosphate was used as a source of P, and unlabelled G was co-purified with G of biosynthetic origin. After substracting 7% of unlabelled, 'degraded' G from the mixture of isotopomers, an average 90.5% of ^{15}N enrichment in the biosynthesized G results.

The ^{1}H-NMR spectrum of **1** in $CDCl_3$ (*Fig. 1a*) shows all signals and coupling constants as expected from unlabelled material. In addition, a coupling in both $H-C(1')$ and $H-C(2')$ resonances to N(9) is visible ($^2J(1',N(9)) \approx 0.5$ and $^3J(2',N(9)) = 1.7$ Hz, resp.). According to steady-state NOE experiments[2]), the resonance at 8.0 ppm is assigned to $H-C(8)$. Its *dd* multiplicity originates from two $^{15}N,^1H$ couplings: $^2J(8,N(9)) = 11.7$ and $^2J(8,N(9)) = 8.3$ Hz. The $H-C(2)$ signal at 8.2 ppm splits into two resonances of unequal intensities. Both show $^{15}N,^1H$ couplings; the larger one is a *dd* with $^2J(2,N(3)) = 13.2$ and $^2J(2,N(1)) = 7.5$ Hz and belongs to the main isotopomer. The smaller one is a *d* with $^2J(2,N(3)) = 13.2$ Hz. The third isotopomer ($^2J(2,1) = 7.5$ Hz), if present, hides underneath the main resonance. The 0.23-Hz difference in *Larmor* frequency between the visible $H-C(2)$ resonances demonstrates a negative (upfield) 92-ppb ^{15}N isotope effect on $H-C(2)$. No third *s* resonance 0.23 Hz downfield of the minor isotopomer is found, in agreement with the low abundancy of unlabelled **1** (0.37% according to MS). The exchangeable $H-N(1)$ resonates at 12.9 ppm as a broad 80-Hz *d*.

In the ^{1}H-NMR spectrum of **2** in $CDCl_3$ (*Fig. 1b*), again various $^{15}N,^1H$-couplings are visible (*e.g.* $^2J(1',N(9)) = 1.2$, $^3J(2',N(9)) = 1.7$, $^3J(2''',N-C(2)) = 0.8$ Hz). The following resonances allow to directly determine the ^{15}N enrichments of most of the N-atoms of **2**. The N-bound protons $HN-C(2)$ and $HN(1)$ resonate at 9.03 and 12.03 ppm, respectively. The coupling constants are $^1J(H,N-C(2)) = 92.5$ and $^1J(H,N(1)) = 91.2$ Hz, respectively. The multiplicity of the $HN-C(2)$ signal is *dd*; the second coupling is interpreted as a long-range coupling to N(3), $^3J(HN-C(2),N(3)) = 2.1$ Hz, since no proton irradiation has any influence on this resonance. ^{15}N Contents of 83% at N(1) and 93% at $N-C(2)$ are calculated from the integrals of the residual *s*.

The $H-C(8)$ resonance of **2** at 7.7 ppm consists of four resonances originating from four isotopomers (*Fig. 2*, bottom). The fully labelled main isotopomer splits its proton resonance into a *ddd* multiplicity without significant roof effect ('*c*' isotopomer in *Fig. 2*, top). $H-C(8)$ couples here with N(7), N(9), and $N-C(2)$ (*sic!*). The weakest coupling can not be influenced by proton irradiations, is absent in **1**, and therefore, originates from a very-long-range $^{15}N,^1H$ coupling over six bonds: $^6J(8,N-C(2)) = 0.8$ Hz. The other two constants are $^2J(8,N(7)) = 11.5$ Hz and $^2J(8,N(9)) = 8.6$ Hz. The other clearly

---

[2]) Conclusive NOE-derived assignments could only be obtained from unlabelled material: Irradiation at the imino resonance $H-^{14}N(1)$ at 13.1 ppm produces a positive 12.5% enhancement at 8.3 ppm and a negative 1.3% enhancement at 8.02 ppm. Upon irradiations at 8.02 or 8.3 ppm, no NOE is observed ar 13.1 ppm, owing to quadrupolar relaxation of the ^{14}N nucleus. However, when labelled **2** is analyzed, strong negative NOE's result, as expected from the negative gyromagnetic ratio of the ^{15}N nucleus ($\gamma(^{15}N) = -2.7126 \cdot 10^7$ *vs.* $\gamma(^{14}N) = 1.9338 \cdot 10^7$ rad/(T·s)), but with opposite intensities: Irradiation at the $H-^{15}N(1)$ resonance at 12.9 ppm leads to +1.0% enhancement at 8.2 and −11.2% enhancement at 8.0 ppm! Conversely, irradiation at 8.2 ppm leads to −1.3% enhancement at 12.9 ppm, whereas irradiation at 8.0 ppm produces −6.0% enhancement at 12.9 ppm. The strong negative NOE between the more distant nuclei $^1H-^{15}N(1)$ and $^1H-C(8)$ must originate from the indirect effect of $^{15}N(1)$ being more or less in-line between $H-N(1)$ and $H-C(8)$. In contrast, the angle $HN(1)-^{15}N-HC(2)$ is unfavorable (*ca.* 90°) [8]. This indirect effect must also have occurred in the unlabelled material, albeit to a smaller extent, since, as at the extreme narrowing limit (as this molecule is), direct enhancements are positive unless there is a strong scalar coupling between the protons (which is not the case). The small positive enhancement of 1% at $H-C(2)$ upon irradiation at $H-^{15}N(1)$, as compared to +12.5% in the unlabelled material, and the small and negative enhancement of −1.3% at $H-^{15}N(1)$ upon irradiation at $H-C(2)$ show that a N(1)-derived indirect effect occurs even in the unfavorable geometry.
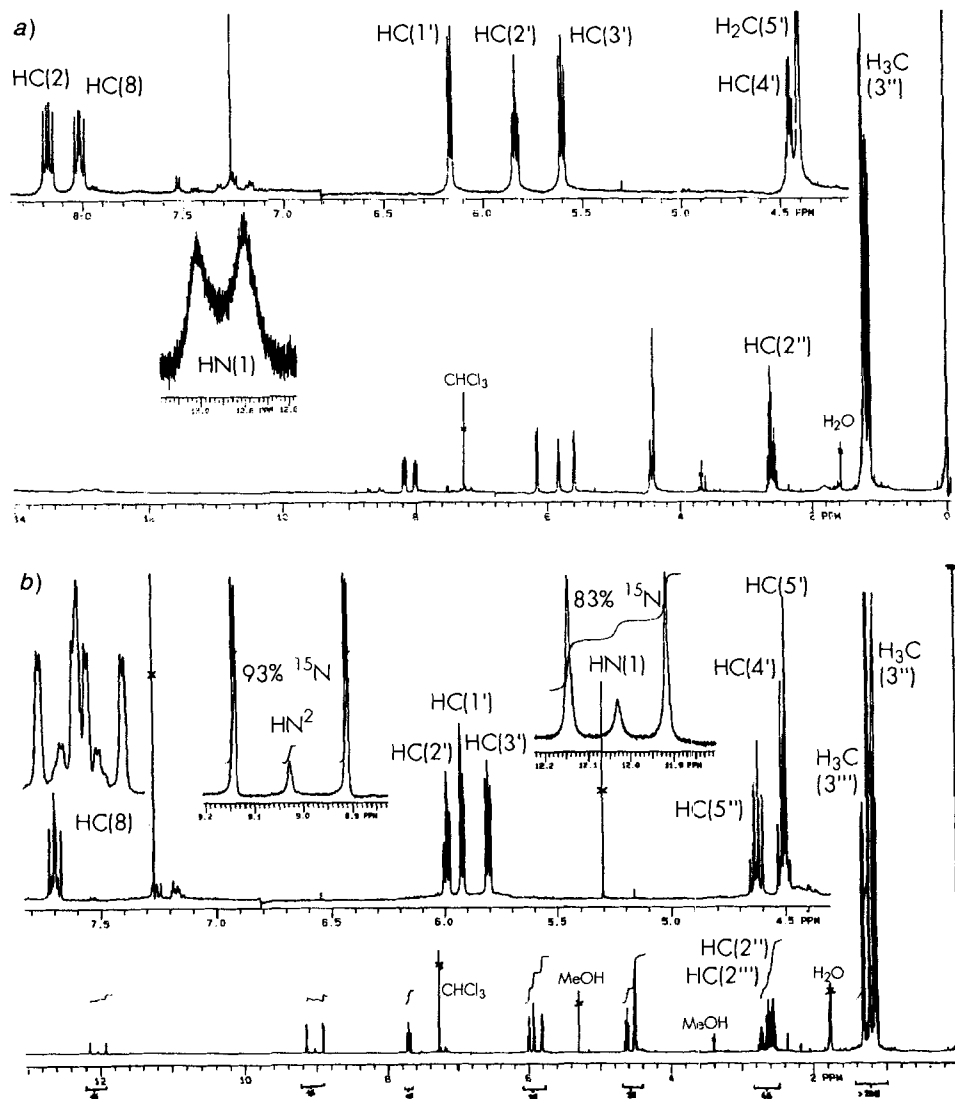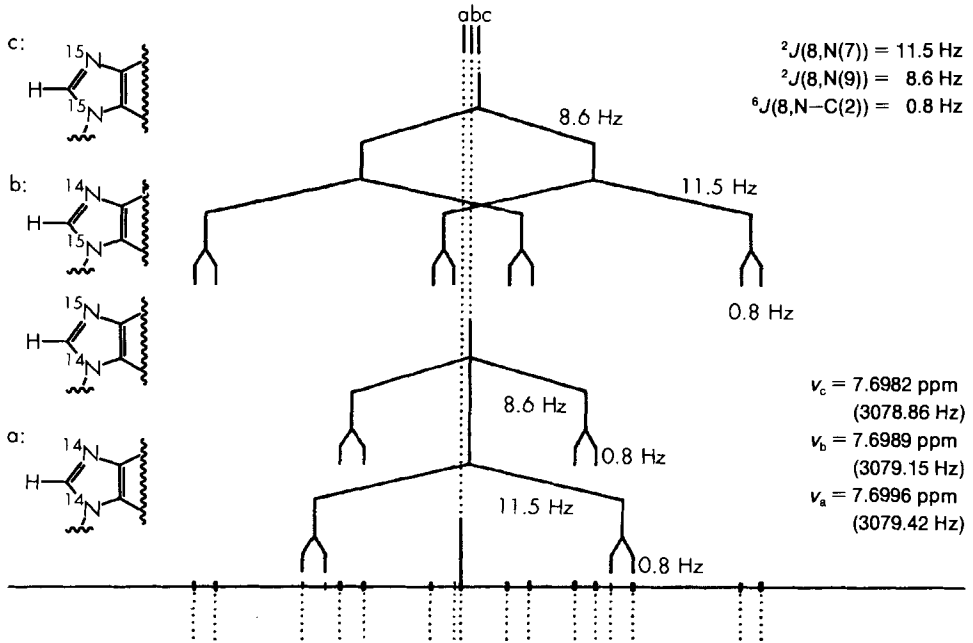
Fig. 1. ^1H-NMR Spectra (400 MHz, CDCl$_3$) of a) **1** and b) **2**. δ rel. to SiMe$_4$ (= 0 ppm).

visible isotopomer resonance is a *dd* with $^2J(8,N(9)) = 8.6$ and $^6J(8,N-C(2)) = 0.8$ Hz (upper 'b' isotopomer in *Fig. 2*, top). Underneath (shoulder), another *dd* resonance ($^2J(8,N(7)) \approx 11.5$ Hz) reveals the presence of a third isotopomer (lower 'b' isotopomer in *Fig. 2*, top). The discrimination of the latter ^2J couplings is possible after inspection of the ^{15}N-NMR spectra (*vide infra*). Finally, a *s* appears slightly downfield of the centre showing the presence of unlabelled material ('a' isotopomer in *Fig. 2*, top). The absence of the 0.8-Hz coupling in this resonance lends further support to the apparent six-bond ^{15}N,^{1}H coupling and confirms that this isotopomer is fully unlabelled. The three kinds of

$^2J(8,N(7)) = 11.5$ Hz
$^2J(8,N(9)) = 8.6$ Hz
$^6J(8,N-C(2)) = 0.8$ Hz

$v_c = 7.6982$ ppm
(3078.86 Hz)
$v_b = 7.6989$ ppm
(3079.15 Hz)
$v_a = 7.6996$ ppm
(3079.42 Hz)

Index	v [Hz]	δ [ppm]	Intensity
01	3089.35	7.724	130.000
02	3088.58	7.722	130.942
03	3083.94	7.711	42.090
04	3083.09	7.709	41.800
05	3080.69	7.703	142.103
06	3079.47	7.700	201.910
07	3077.82	7.696	140.188
08	3077.01	7.693	133.642
09	3075.28	7.689	39.539
10	3074.42	7.687	38.694
11	3073.06	7.684	14.547
12	3069.13	7.674	130.088
13	3068.38	7.672	127.809
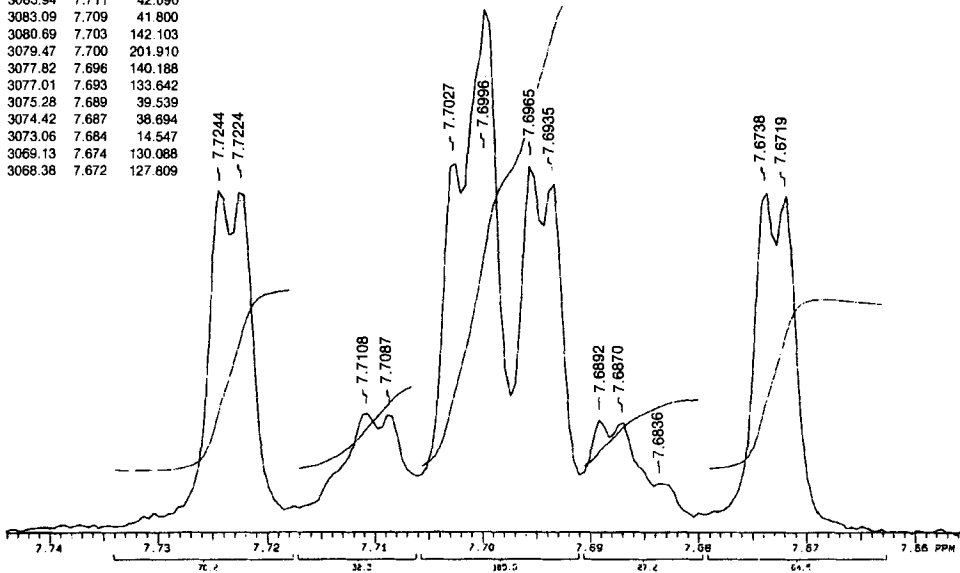
Fig. 2. *Close-up of the H−C(8) ^{1}H-NMR signal of* **2**

isotopomers 'c', 'b', and 'a', one carrying two ^{15}N atoms in positions 7 and 9, two carrying only one ^{15}N, and the unlabelled one, resonate at significantly different *Larmor* frequencies. The ^{15}N isotope effect is 112 ppb (0.28 Hz) per ^{15}N atom for this proton.

The integral ratio between the leftmost *d*'s of the 'c' and 'b' isotopomers is 68:32. The ratio between both 'b' isotopomers is estimated to be approximately ^{15}N(9)/^{15}N(7) 2:1 to 3:1. The 'b' and 'c' isotopomers together correspond to 93% ^{15}N, leaving 7% to the unlabelled 'a' isotopomer (MS). The ^{15}N content multiplied by the ratio yields ^{15}N(9),^{15}N(7)/^{15}N(9),^{14}N(7)/^{14}N(9),^{15}N(7)/^{14}N(9),^{14}N(7) *ca.* 63:22:8:7. Hence, the ^{15}N enrichment of all N-atoms can be derived from the combined ^{1}H-NMR and MS analysis (the enrichment at N(3) can be calculated from the average ^{15}N content of 83.5%). Since inosine is the biosynthetic precursor of guanosine and has an average ^{15}N content of 91.9%, the corresponding ^{15}N enrichments can also be calculated. *Table 4* summarizes the results.

Table 4. ^{15}N Enrichment in All N-Positions of Biosynthesized Guanosine and Inosine

Order of biosynthesis	Atom	Approx. ^{15}N enrichment		Biosynthetic precursor
		G [%]	I [%]	
1	N(9)	85	96	Gln ($\rightarrow$ Glu)
2	N(7)	71	80.5	Gly
3	N(3)	85.5	97	Gln ($\rightarrow$ Glu)
4	N(1)	83	94	Asp ($\rightarrow$ fumarate)
5	N^2	93	–	Gln ($\rightarrow$ Glu)
	average	83.5	91.9	

The specific enrichments are in accordance with the biosynthetic pathway to inosine and guanosine. The first N-atom to be introduced is N(9) and stems from the side chain of glutamine. It is attached to α-D-5-phosphoribofuranosyl 1-pyrophosphate to form β-amino-D-ribofuranosyl 5'-phosphate. The imidazole ring of purine nucleosides is built up through the condensation of glycine to N(9), N(7)-formylation involving N^{10}-formyltetrahydrofolate (N^{10}-formyl-THF), transfer of a glutamine side chain N-atom onto the resulting C(4)-carbonyl group, and subsequent eliminative cyclization (C(8)–N(9)). Thus, the second N-atom is N(7) and the third N(3). The resulting 5-aminoimidazole ribonucleotide is carboxylated at C(5) and condensed with aspartate. Only the N-atom of aspartate remains in the purine as N(1), the fourth N-atom (the rest of aspartate eliminates as fumarate). Subsequent N(3)-formylation and eliminative cyclization yields inosinate, the common biosynthetic precursor of guanylate and adenylate. C(2) is oxidized to form xanthylate which is transaminated to guanylate through the introduction of N–C(2), derived, one again, from the side chain of glutamine.

THF = tetrahydrofolate
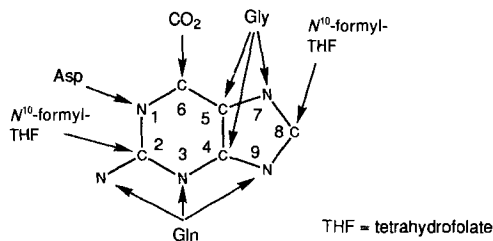
According to *Table 4*, most of the glutamine had to be biosynthesized during culture growth, since the enrichment of N(9) in inosine is already quite high. As expected, the enrichment of N(3) is of the same order. The enrichment of N—C(2) of guanosine is lower only due to the 7-% fraction of fully unlabelled guanosine of exogeneous origin. However, unlabelled glycine must have been quite abundant in the culture medium, since only 80.5% of N(7) originates from biosynthesized glycine. In fact, there was even more unlabelled glycine present than added unlabelled aspartate, N(1) being almost as enriched as the glutamine-derived N-atoms (94%). Finally, at the stage where xanthylate is transaminated to guanylate, only biosynthesized glutamine was present in the culture medium.
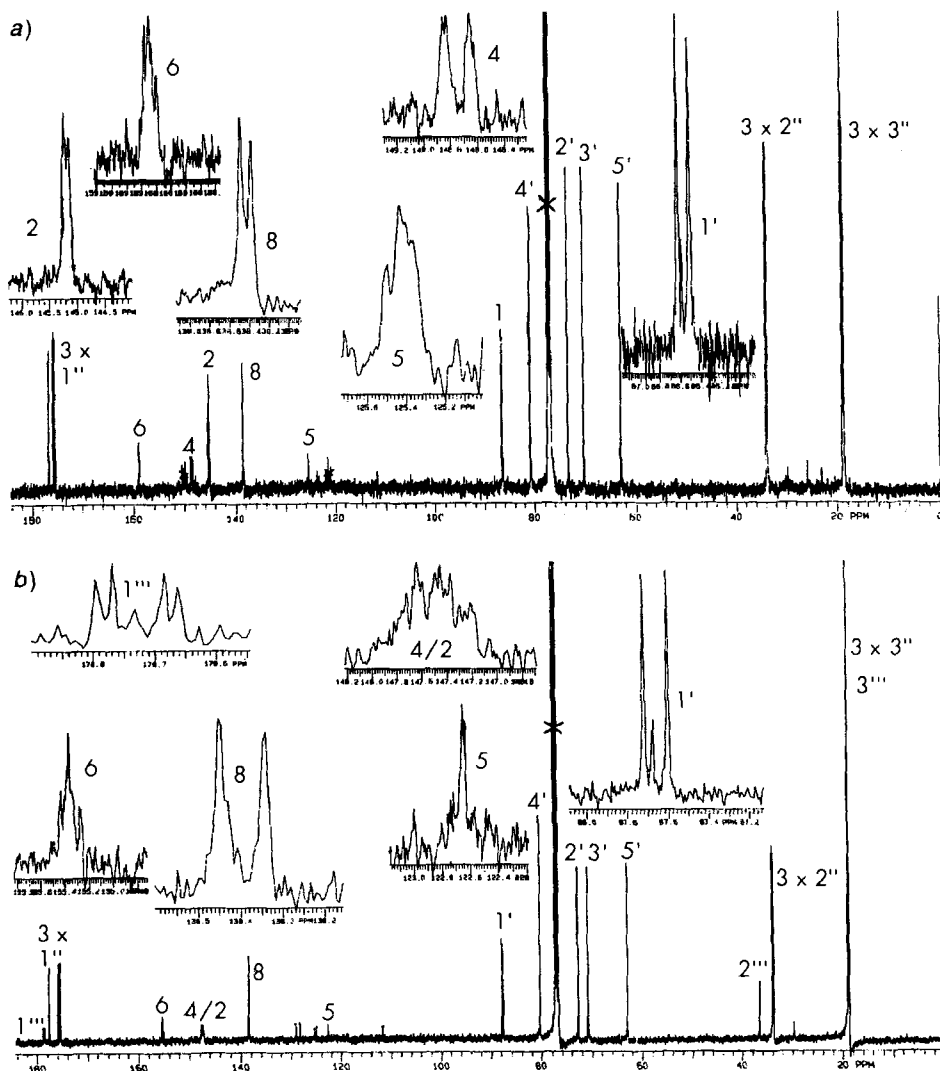
Fig. 3. *^{1}H-Broad-band-decoupled ^{13}C-NMR spectra* (101 MHz, CDCl$_3$) *of* a) **1** *and* b) **2**. δ rel. to SiMe$_4$ (= 0 ppm).

To learn more about $^{13}C,^{15}N$ couplings, ^{13}C-NMR spectra of **1** and **2** were measured (*Fig. 3*). The $\delta(^{13}C)$ agree well with published data, the resonance splittings due to ^{15}N-couplings, however, are sometimes rather difficult to explain. Especially the known facts that long-range coupling constants may be larger than short-range coupling constants and that some couplings simply do not occur [9] render the assignments of couplings somewhat intuitive. Nevertheless, some conclusive information can be elucidated. In particular, the $C(1')$ resonances do couple with $N(9)$, whereas $C(2')$ resonances do not any more. The constants are the same in both compounds: $^2J(N(9),C(1')) = 12$ Hz. The non-labelled isotopomers appear in the $C(1')$ signals of both compounds as central, slightly downfield-shifted *s*'s. The ^{15}N isotope effect on $C(1')$ is -172 ppb in **1** and -131 ppb in **2**. The well-resolved $C(1')$ signals of **2** show an intensity ratio $^{15}N(9)/^{14}N(9)$ of 85:15, in excellent agreement with the value derived from ^1H-NMR. The other measurable ^{15}N isotope effect is -66 ppb on $C(1''')$ of **2**. Some of the other $^{15}N,^{13}C$-couplings in the bases are less complex, others, however, are more complex than expected. All visible $^{15}N,^{13}C$-couplings, together with the sometimes tentative assignments, are described in the *Exper. Part*.

Finally, ^1H-coupled and broad-band-decoupled ^{15}N-NMR spectra of **1** and **2** in $CDCl_3$ were measured (*Fig. 4*). All $\delta(^{15}N)$ and signal intensities are consistent with literature data [10]. The spectrum of **1** shows four signals: $N(9)$ at -210.6, $N(1)$ as a broad *m* at -208.4, $N(3)$ at -163.9, and $N(7)$ at -133.3 ppm (*Fig. 4a*). The resonances are assigned according to selective ^1H irradiations. All $^1H,^{15}N$- and $^{15}N,^{15}N$ couplings are listed in the *Exper. Part*. Most importantly, the couplings between $H-C(8)$ and $N(9)$ (8.2 Hz), $N(7)$ (11.6 Hz) and between $H-C(2)$ and $N(3)$ (13.2 Hz) precisely agree with the couplings and assignments derived from the ^1H-NMR data.

The spectrum of **2** shows five resonances with expected chemical shifts and signal intensities: $N-C(2)$ at -246.7, $N(1)$ at -228.2, $N(9)$ at -213.2, $N(3)$ at -200.0, and $N(7)$ at -132.8 ppm (*Fig. 4b*). All $^1H,^{15}N$-coupling constants derived from the ^1H-NMR spectrum agree well with the ^{15}N-NMR data (see *Exper. Part*). The small long-range couplings between $H-C(1')/H-C(2')$ and $N(9)$ (1.2 and 1.7 Hz, resp.) can be observed in fully ^1H-coupled spectra, but cannot be quantified due to additional $^{15}N,^{15}N$ couplings. The largest $^{15}N,^{15}N$-coupling constant of 16.8 Hz occurs between $N-C(2)$ and $N(1)$. $N-C(2)$ additionally couples with $N(3)$ (6.9 Hz). $N(3)$, in turn, couples with $N(9)$ (3.5 Hz). Both $N(9)$ and $N(3)$ resonances show an additional splitting of 1.5 Hz which can not be found in any other signal and cannot be influenced by proton irradiations. In contrast to $N(7)$ of **1** ($^2J(N(7),N(9)) = 2$ Hz), $N(7)$ of **2** appears as a *s* apparently having a zero-coupling with $N(9)$. Note that, in order to reliably determine $^{15}N,^{15}N$-coupling constants, the frequency of the ^1H decoupler has to correspond precisely to the proton frequency of the proton to be decoupled, despite of the 'super-efficiently' broadening WALTZ puls sequence.

A small part of compound **2** was deprotected with ammonia to give $[N^2,1,3,7,9-^{15}N]$-guanosine. The ^1H-broad-band-decoupled ^{15}N-NMR spectrum in phosphate-buffered water, pH 7.0, shows five resonances (chemical shifts relative to internal $^{15}NH_4Cl$): $N-C(2)$ at 51.7, $N(1)$ at 127.2, $N(3)$ at 145.2, $N(9)$ at 147.9, and $N(7)$ at 214.95 ppm. In addition, preliminary experiments were carried out with unlabelled $ib^2Gib_3^{2',3',5'}$, in order to synthesize $[O^6-^{17}O]$guanosine. Thus, $ib^2Gib_3^{2',3',5'}$ was converted into N^2-isobutyryl-2',3',5'-tri-O-isobutyryl-O^6-(2''',4''',6'''-triisopropylphenylsulfonyl)guanosine   ($ib^2tps^6Gib_3^{2',3',5'}$)
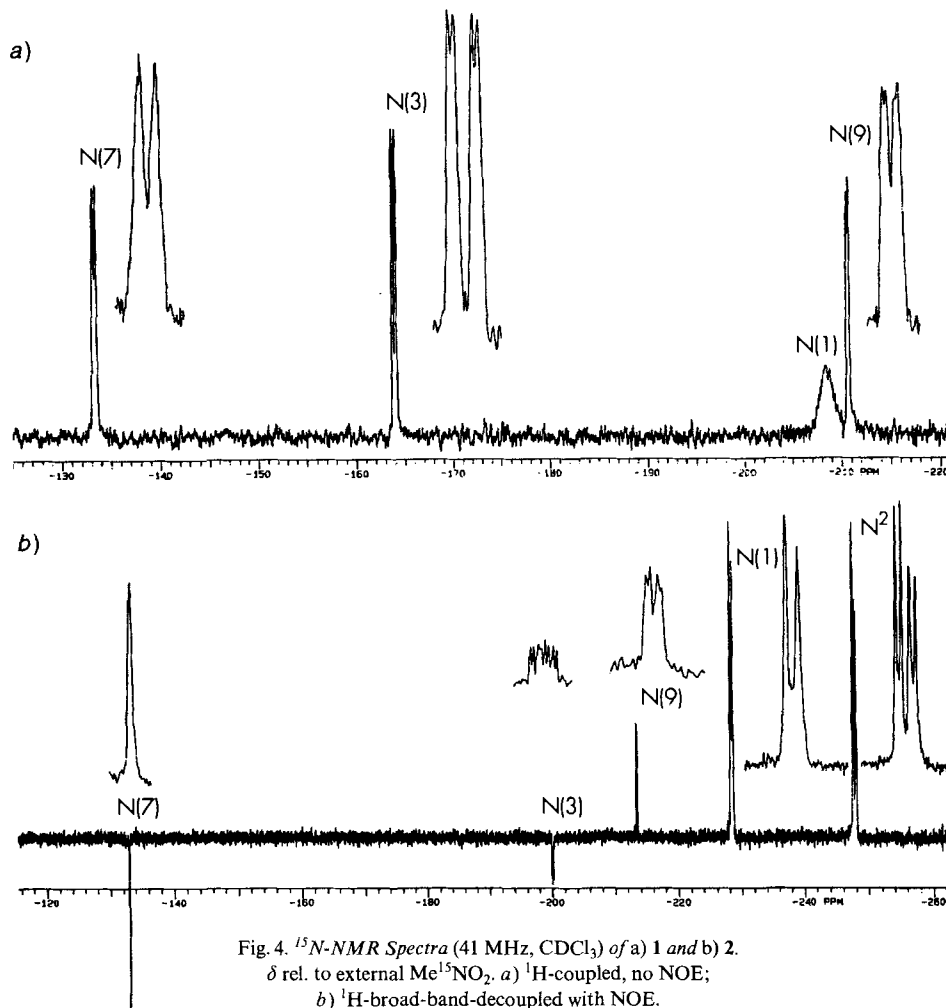
a)

b)

Fig. 4. ^{15}N-NMR Spectra (41 MHz, CDCl$_3$) of a) **1** and b) **2**.
δ rel. to external Me^{15}NO$_2$. a) ^{1}H-coupled, no NOE;
b) ^{1}H-broad-band-decoupled with NOE.

[11]. In the presence of Me$_3$N and a strong base, the sulfonate was hydrolyzed with labelled water. Subsequent deprotection with ammonia furnished [O^6-^{17}O]guanosine (10 atom-% ^{17}O). The ^{17}O-NMR spectrum of [O^6-^{17}O]guanosine in buffered water at 45° shows a signal at 245.3 ppm with a half-intensity-width of 725 Hz (chemical shift relative to external 1,4-dioxane). Both heteronuclear NMR spectra will serve as a reference for ^{15}N- and ^{17}O-labelled RNA.

 [N^6,1,3,7,9-^{15}N]Adenosine. The conversion of **1** into [N^6,1,3,7,9-^{15}N]adenosine involved two steps, activation of the base carbonyl group through the introduction of a leaving group and substitution with labelled ammonia. The introduction of the 2,4,5-triisopropylphenylsulfonyl (tps) group into **1** under the same conditions as above furnished a 1:1 mixture of two isomers. The ^{1}H-NMR spectrum of the purified mixture showed that both O^6 and N(1) atoms of **1** reacted unspecifically with the reagent. In addition, the

reaction of similar O-sulfonate derivatives with small nucleophiles such as ammonia or water can be unselective with respect to the attack at the S- vs. the C-centre (unpublished results).

Previously, 3-nitro-1,2,4-triazole was shown to be a useful leaving group when bound to C(4) of a thymidine derivative; diphenyl phosphorochloridate was used as a condensing reagent [12]. However, when 1 was submitted to these reaction conditions, the corresponding (intermediate) O^6-(diphenyl-phosphate) derivative was too stable to be substituted either by triethylammonium 3-nitro-1,2,4-triazolide or ammonia. The nitrotriazolyl derivative of 1 did form upon the action of phosphoryltris(3-nitro-1H-1,2,4-triazole), albeit in low yields.

The best way to activate 1 was to use phosphoryl-tris(1,2,4-triazole) [13]. A 6:1 mixture of the stable, isomeric compounds 3a/b (Scheme 2) was obtained in 80% yield (not optimized). The ^{1}H-NMR spectrum of the purified product showed that the major isomer was the nonsymmetrical triazolyl derivative 3a. Ammonolysis of the mixture with 20% aqueous $^{15}NH_3$ solution in bi-phasic H_2O/tetrahydrofuran furnished 4 in 90% yield. No trace of inosine owing to a competing hydrolysis of 3a/b was found (TLC, reversed-phase MPLC).

Scheme 2. Synthesis of [N^6,1,3,7,9-^{15}N] Adenosine
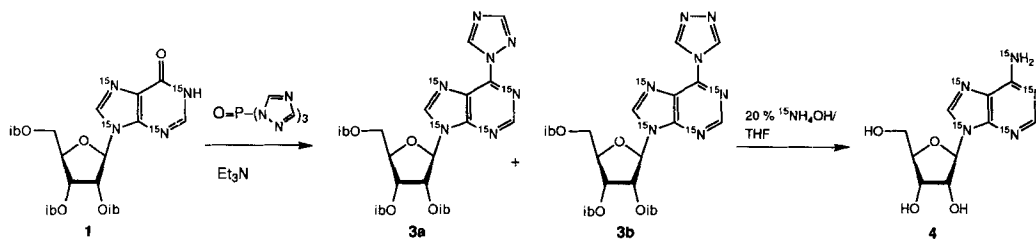
The FAB$^+$-MS of 4 shows the expected protonated molecular- and base-ion signals at m/z 273 and 141, respectively. The ^{1}H-NMR spectrum in CD$_3$OD exhibits all expected resonances. The H−C(2') resonance, as opposed to the H−C(1') resonance, shows an unusually high long-range coupling with N(9) ($^3J(2',N(9)) \approx 4.6$ Hz, $^2J(1',N(9)) = 1.4$ Hz). In contrast to the H−C(2) resonance of 1 (dd), $J(N(1),H$−C(2)) of 4 is identical to $J(N(3),H$−C(2)) producing a t of 14.8 Hz at 8.18 ppm. The minor ($^{15}N(3),^{14}N(1)$)-isotopomer shows as a small 14.8 Hz-d. H−C(8) resonates at 8.30 ppm with similar $^{15}N,^1$H-coupling constants as in 1 ($^2J(8,N(9)) = 8.0$, $^2J(8,N(7)) = 11.3$). The ^{1}H-broadband-decoupled ^{15}N-NMR spectrum of 4 in phosphate-buffered water, pH 7.0, exhibits five resonances at the expected frequencies (chemical shifts relative to internal $^{15}NH_4Cl$): N−C(6) at 57.2, N(9) at 148.2, N(3) at 197.7, N(1) at 206.6, and N(7) at 210.8 ppm.

Conclusion. – The biosynthetic method developed by Pardi and coworkers produced ca. 80–100 µmol of each mononucleotide per litre culture medium (from E. coli RNA) [3], the method by Williamson and coworkers ca. 1 µmol of each mononucleotide (from Methylophilus methylotrophus RNA) [4]. The aim in these studies was to introduce ^{13}C isotopes into the mononucleotides as well, by feeding E. coli with [U-^{13}C]glucose and M. methylotrophus with less expensive ^{13}CH$_3$OH. In addition, one litre contained 0.68 and 0.78 g of ($^{15}NH_4)_2SO_4$, respectively.

The presented approach necessitated ca. 9 g of ($^{15}N_2$)urea and 19 g of ($^{15}NH_4)_2SO_4$ per litre. From the residual culture broth, 5.85 g of $^{15}NH_4Cl$, corresponding to ca. 36% of added $^{15}NH_4^+$ ions or 18% of the total amount of ^{15}N, could be regenerated by H_2O-vapour distillation. In terms of yield per consumed equivalent ^5N, Pardi and coworkers

obtained 0.87% of AMP and 1.07% of GMP, *Williamson* and coworkers 0.058% of AMP and 0.085% of GMP, and 1.68% of inosine and 5.14% of guanosine (as determined by HPLC). Hence, *E. coli* and *B. subtilis* are comparably efficient in incorporating N into nucleobases, while *M. methylotrophus* lags behind by a factor of more than 10.

A guanosine- and inosine-overproducing strain has the advantage of a rather simple isolation and purification procedure, because the metabolites are being released into the culture medium. The method could be up-scaled even further to produce multigramme quantities of ^{15}N-labelled purine nucleosides, be it for *in vitro* transcription or chemical RNA synthesis.

Experimental Part

General. Pyridine was refluxed (12 h) and distilled over CaH_2. ($^{15}N_2$)Urea, $^{15}NH_4Cl$, and $Me^{15}NO_2$ (99% ^{15}N) were purchased from *Cambridge Isotope Laboratories*. Thin-layer chromatography (TLC): Silica gel *60 F$_{254}$* (*Merck*); detection under UV light, I_2, and by dil. methanolic H_2SO_4 followed by 2% (*w/v*) ethanolic naphthoresorcinol and heating. Column chromatography (CC) and flash chromatography (FC): silica gel *60*, 63–200 μm and 40–63 μm, resp. (*Merck*); for large scale FC, silica gel *C 560*, 35–70 μm (*Uetikon*). Medium-pressure liquid chromatography (MPLC, max. 40 bar; *Büchi*), programmed low-pressure gradient mixing, UV detection with prep. flow-cell and variable wavelength (*Kontron*); reversed-phase column (*Büchi*): 46 × 3.6 cm i.d. containing 250 g of *LiChroprep® RP-18* (*Merck*), 15–25 μm (packed as toluene suspension); normal-phase column (*Büchi*): 46 × 7.0 cm i.d. containing 1 kg of *LiChroprep® Si 60*, 15–25 μm (packed as toluene suspension). High-performance liquid chromatography (HPLC): *Shimadzu IC-7A*, high-pressure gradient mixing, column oven, UV detection at variable wavelength (*Uvikon 722* LC spectrophotometer, *Kontron*), digital peak integration (*HP 3380 A, Hewlett-Packard*); anal. HPLC: *LiChrosorb® RP-18*, 5 μm, 50 Å, 250 × 4 mm i.d. (*Merck*); semi-prep. HPLC: *ODS II*, 5 μm, 50 Å (*Spherisorb*), 250 × 16 mm i.d. (*Knauer*). Anion-exchange chromatography: *Amberlite® CG-400 II*, 200–400 mesh, Cl⁻ form (*Fluka*). Cation-exchange chromatography: *Chelex 100®*, 200–400 mesh, Na⁺ form (*Biorad*). M.p. (corrected): *Kofler* block. NMR Spectra: *Varian-Gemini-300* and *Varian-VXR-400*; δ(H), δ(C), δ(N), and δ(O) in ppm rel. to internal or external standards, as indicated; scalar coupling constant *J* in Hz. ^{15}N-NMR Spectra: 10 mm o.d. sample tube; spectral width 25 kHz, acquisition time 640 ms, pulse width 90° (21.0 μs), pre-acquisition delay 5.0 s, ^{1}H-broadband decoupling with WALTZ sequence; double-precision 65536 B-*Fourier* transformation with zero *K* filling, no line broadening. ^{17}O-NMR Spectra: 10 mm o.d. sample tube; spectral width 50 kHz, acquisition time 20 ms, pulse width 90° (45.0 μs), no pre-acquisition delay, no ^{1}H decoupling; no ^{2}H lock; double-precision 65536 B-*Fourier* transformation with zero *K* filling, line broadening 100 Hz (half-intensity signal width is given after subtraction of the line broadening). MS *VG 70-250*; fast-atom bombardement (FAB, Xe), matrix nitrobenzyl alcohol (NBA) or glycerine (glyc); mass peaks in *m/z* (rel. intensity in %; FAB intensities after subtraction of the matrix spectrum).

Purification of $^{15}NH_4Cl$ and Conversion into Pure ($^{15}NH_4$)$_2SO_4$. An aq. soln. (200 ml) of $^{15}NH_4Cl$ (20.05 g, 0.37 mol) was extracted with CH_2Cl_2 (3 × 50 ml). The combined org. extracts were washed with H_2O (50 ml). The combined aq. solns. were concentrated *in vacuo* to saturation (*ca.* 50 ml), transferred into a *Parnas-Wagner* H_2O-vapour distillation apparatus (*ca.* 100-ml volume) [14], and treated with NaOH (30 g, 0.75 mol) in H_2O (25 ml). During heating and distilling, the resulting $^{15}NH_4OH$ soln. was captured in a vessel containing H_2SO_4 (8.3 ml, 0.3 mol; *ca.* 80% of the amount needed) in H_2O (200 ml). During the distillation, the pH of the stirred ($^{15}NH_4$)$_2SO_4$ soln. was continuously measured and kept below 6.5 by addition of 2N H_2SO_4. The final pH was 5.2. The distillation was continued for further 10 min (constant pH). The ($^{15}NH_4$)$_2SO_4$ soln. was lyophilized overnight

to yield 25.32 g (103%) of (^{15}NH$_4$)$_2$SO$_4$. A 1N soln. thereof was passed over a column ($\varnothing$ 3 cm, h 5 cm) filled with pretreated *Chelex-100* (15 g, flow rate 20 ml min^{-1}). The resin was washed with H$_2$O (200 ml). After lyophilization overnight, (^{15}NH$_4$)$_2$SO$_4$ (25.72 g, 104%), free of heavy metals, was obtained (4% Na$_2$SO$_4$). The resin was regenerated with 2 column volumes (h 5 cm) of 1N HCl (prepared from distilled 6N HCl), 2 volumes of 1N NaOH and 5 volumes of H$_2$O. The HCl soln. was concentrated to 100 ml and used for MS analyses.

   Purification of (^{15}N$_2$) Urea. A 0.15 M aq. (^{15}N$_2$)urea soln. was passed over *Chelex-100* (30 g). The resin was washed with 1 l of H$_2$O as described above (except h 10 cm). Lyophilization overnight furnished 10.52 g (101%) of (^{15}N$_2$)urea free of heavy metals. The resin was regenerated as described above.

   Microbiological Methods and Biosynthesis. Nutrient media: *Luria-Bertani* (LB) medium ([15], modified): *Bacto* tryptone (10 g; *Difco Laboratories*, Detroit, Michigan, USA), yeast extract (5g; *Difco*), NaCl (10 g), agar (15 g), adenine (20 mg), H$_2$O (1000 ml); the pH was adjusted to 7.5 with 2N NaOH prior to sterilization. SD Medium [6]: D-sorbitol (20 g; *Sigma*), autolyzed yeast (15 g; *Difco*), KH$_2$PO$_4$ (1 g), K$_2$HPO$_4$·3H$_2$O (3 g), H$_2$O (1000 ml); the pH was adjusted to 6.5 with 1N HCl prior to sterilization. The FM medium was prepared in two portions: anh. D-glucose (100 g; *Siefried*, Zofingen, CH) was added to H$_2$O (300 ml) and sterilized (FM a). Separately, CaCO$_3$ (30 g; *Fluka purum*), (^{15}NH$_4$)SO$_4$ (18.92 g), (^{15}N$_2$)urea (9.32 g), L-aspartic acid (0.8 g; *Fluka puriss.*), corn steep liquor (20 g; *Sigma*), *T. utilis* RNA (2.0 g; *Fluka*), CaCl$_2$·6 H$_2$O (5.0 g; *Fluka BioChemika*), MgSO$_4$·7 H$_2$O (2.0 g; *Fluka puriss., p.a.*), KCl (0.5 g; *Merck p.a.*), MnSO$_4$·4–6 H$_2$O (2.5 mg; *Fluka puriss, p.a.*) were added to H$_2$O (700 ml), adjusted to pH 6.8 with 2N NaOH and autoclaved (FM b). All media were kept under cotton plugs and stored at 4° if necessary.

   B. subtilis is pathogenic to humans and should be handled with appropriate caution [16]. Stock cultures of strain NA6601 were maintained on agar slants containing *LB* medium (3 ml each). After incubation for 72 h at 28°, these were stored at 4° and used to inoculate fresh slants every two months. For the biosynthetic production, a portion of the stock was first transferred onto fresh *LB* medium and incubated at 28°. A 1-ml portion of SD medium was added to the slant and used to suspend the bacteria. The suspension was then transferred into a creased 300-ml *Erlenmeyer* flask containing 30 ml of SD medium. The SD cultures were incubated at 37° in a bench-top rotary shaker (270 rpm) for 24 h in the dark. For the production, creased 500-ml *Erlenmeyer* flasks were used each containing FM b (14 ml), FM a (6 ml), and added incubated SD medium (6.5 ml, 33% v/v)^3). These were incubated at 37° for 80 h in the dark with a steady flow of O$_2$ (*ca.* 1 ml min^{-1}) into the chamber of the rotary shaker.

   Occasionally, some cultures became spontaneously unstable with respect to inosine and guanosine production. Several different stocks were therefore kept and used in parallel. In a typical production of ^{15}N-labelled nucleosides, 3 stocks were used to inoculate 15 fresh *LB* slants. Then 3 *LB* cultures of different origin were transferred into five 30-ml portions of SD medium in creased 300-ml *Erlenmeyer* flasks. After the usual incubation, the latter were used to inoculate 14 portions (20 ml each) of FM medium.

   In most FM cultures, part of the bacteria began to stick to the glass surface above the broth after some h forming a brown ring. Occasionally, this ring was washed back by tilting the *Erlenmeyer* flasks by hand (5–10 times within 80 h). At the same time, a foam appeared over many cultures, which was a good indicator for the future nucleoside production, unless it grew up to 2-cm thick. If this was the case, the foamy flasks were taken out every few h into r.t. allowing the foam to collaps during a 10 min pause. These flasks turned out to be the best producers. The SD cultures produced the rings as well, but never foamed. The decrease in volume of the 26.5-ml cultures due to evaporation during the 80-h period was 1.5–2.5%.

   Reversed-phase HPLC Analysis of Inosine and Guanosine. Buffer A : 0.2M NH$_4$(HCOO), pH *ca.* 3.5; buffer B : A/MeOH 7:3. Flow rate 1.3 ml min^{-1}. Time programme: 0–1 min 100% A, 1–15 min 100→50% A (linear), 15–15.2 min 50→100% A, 15.2–23 min 100% A. Temp. 40°. Detection at 259 nm. The culture broth (0.1 ml) was mixed with buffer A (10 ml) and filtered through 1 µm, then 0.2 µm pores. Per run, 10 µl were injected. t_R (I) 9–11 min, t_R (G) = t_R (I) + 0.8 min. No volume correction factor owing to evaporation was applied for the peak integrals.

   Workup and Formation of Derivatives 1 and 2. Celite (150 g) was added to the culture broth (850 ml). The mixture was stirred and boiled for 10 min. The suspension was filtered hot through a vapour-heated funnel (*Whatman GF*). The precipitates were boiled with H$_2$O (100 ml) and filtered. HPLC Analysis showed the absence of I and G in the residual precipitates. The filtrates were concentrated and cooled to 0°. Subsequent filtrations, concentrations of the mother liquors, and coolings furnished up to 76% of crude G and smaller fractions of I (HPLC). The final crop was obtained after extracting the mother liquor with CH$_2$Cl$_2$ (3 × 100 ml). A total amount

---

^{3})   FM cultures of twice and four-fold volumes, as well as 1000-ml cultures in an aerated fermenter with or without pH control produced less, less reliably, or not at all. In general, the smaller the volume, the higher was the yield. For guanosine production, 53-ml portions proved to be equally good as 26.5-ml portions, but more inosine was produced in the smaller portions.

of 16.8 g of off-white solid was obtained after drying *in vacuo*. The noncrystallizable final mother liquor was enriched in I. It was dissolved in H_2O, carefully adjusted to pH 11 and rapidly vapour-distilled in the *Parnas-Wagner* apparatus during 10 min, to regenerate unused $^{15}NH_3$ as its hydrochloride. The residual soln. was readjusted from pH 10 to 11, and the resulting clear soln. (150 ml) was applied onto a FC column containing strong anion-exchange resin (50 g; $\varnothing$ 3 cm, *h* 10 cm). The resin, originally in the Cl⁻ form, had been pretreated with H_2O (100 ml) for 3–4 h, packed into the column, and washed with 2N NaOH (200 ml) and H_2O until the eluate was neutral. After adsorption of the mother liquor onto the resin, it was first washed with H_2O until the pH was neutral (500 ml). According to HPLC, this soln. was free of G and I. Subsequently, 5% aq. AcOH soln. was used to elute and fractionate (40 × 25 ml) acidic compounds, among them I and G. The I- and G-containing fractions (Nos. 3–23) were pooled, concentrated *in vacuo*, and lyophilized to yield 4.33 g of brownish solid.

Both solids were resuspended in dry pyridine and co-evaporated under reduced pressure (3 × 50 ml per 5 g of solid). Isobutyryl chloride (30 ml, *Fluka*) was added to a stirred 5–10% suspension in dry pyridine (200 ml) at 0°. After stirring overnight at r.t., MeOH (100 ml) was added to the cooled clear soln. and, after 30 min at r.t., the solvents were evaporated. The resulting reddish amorphous solid was dissolved in Et_2O and extracted with 5% aq. $NaHCO_3$ soln. and brine. The aq. extracts were washed with small amounts of CH_2Cl_2 and the combined org. phases dried (Na_2SO_4) and evaporated. Thus, 12.4 g of G-enriched solid gave 17 g of crude 1/2, whereas 24 g of crude 1/2 were obtained from 5.5 g of I-enriched solid.

Reversed-phase HPLC Analysis of 1 and 2. Eluant *A*: H_2O/MeOH 1:1, eluant *B*: MeOH. Flow rate 2 ml min⁻¹. Time programme: 0–0.5 min 0%B, 0.5–1.5 min. 0→30% *B* (lin.), 1.5–6.5 min 30→40% *B* (lin.), 6.5–11.5 min 40→60% *B* (lin.), 11.5–15 min 60% *B*, 15–15.1 min 60→0% *B*, 15.1–20 min 0% *B*. Temp. 40°. Detection at 259 nm. t_R (1) *ca.* 13 min, t_R (2) *ca.* 16 min.

Chromatographic Purification and Separation of 1 and 2. Ca. 6 g of crude 1/2 were submitted to FC SiO_2 (750 g, $\varnothing$ 10 cm, *h* 40 cm). Fractions X (containing 2 and an unidentified compound in large amounts but only in one batch), A, B, and C were separately purified by reversed-phase MPLC. The fractions were each dissolved in CH_2Cl_2, mixed with reversed-phase SiO_2 from 3 precolumns and evaporated to complete dryness. The impregnates were resuspended in H_2O/MeOH 1:1 (reversed-phase MPLC eluant *A*) and pressed into the mounted and empty precolumns. After conditioning with eluant *A* for 10 min at the maximum flow rate, the gradient was started (eluant *B* = MeOH): 1 min *A*, 15 min 0–50% *B*, 30 min 50–75% *B*, 15 min 75–100% *B*, 20 min 100% *B*, 1 min 100–0% *B*, 20 min 0% *B*. The pressure was kept at 38–40 bar, *i.e.*, the flow rate was continuously readjusted as the pressure decreased with increasing MeOH contents (5–10 ml min⁻¹). The fractions were analyzed by reversed-phase HPLC and pooled according to their composition. A total of 5.6 g of G fraction and 4.0 g of I fraction were obtained as yellowish foams. The G fraction was dissolved in CH_2Cl_2(10 ml), loaded onto the normal-phase MPLC column, and chromatographed with 4% MeOH in CH_2Cl_2. The fractions were analyzed by TLC. The regeneration of the column involved a washing step with 8% MeOH/CH_2Cl_2 (4 l), reconditioning with CH_2Cl_2 (1.5 l) and 4% MeOH/CH_2Cl_2 (500 ml). The I fraction was dissolved in 10 ml of this solvent and chromatographed with 6% MeOH/CH_2Cl_2. The column was subsequently washed with 8% MeOH/CH_2Cl_2 (2 l). The separations yielded 5.04 g of 2 and 1.44 g of 1.

Spectroscopic Characterization of 2',3',5'-Tris-O-(2-methylpropanoyl) [1,3,7,9-¹⁵N]inosine (1). ¹H-NMR (400 MHz, CDCl₃, SiMe₄): 1.136, 1.162, 1.18–1.24 (2d, *J* = 7, and *m*, resp., 3 *Me₂CHCOO*); 2.57, 2.63 (2*sept.*, *J* = 7, 1 + 2 H, 3 Me₂*CH*COO); 4.41 (d, *J* = 3.7, CH₂(5')); 4.46 (*td*, *J* = 3.8, 3.9, H–C(4')); 5.60 (dd, *J* = 5, 3.8, H–C(3')); 5.85 (*td*, *J* = 5.4, ³*J*(H–C(2'),N(9)) = 1.7, H–C(2')); 6.16 (*d*, *J* = 5.1, ²*J*(H–C(1'),N(9)) ≈ 0.5, H–C(1')); 8.02 (*dd*, ²*J*(H–C(8),N(7)) = 11.65, ²*J*(H–C(8),N(9)) = 8.3, H–C(8)); 8.17 (*d* + dd, ²*J*(H–C(2),N(3)) = 13.2, ²*J*(H–C(2),N(1)) = 7.5, H–C(2)); 12.90 (*d*, ¹*J*(*H*N(1),N(1)) = 83, HN(1)). ¹³C-NMR (101 MHz, CDCl₃, SiMe₄): 18.7–19.0 (6*s*, 6 C(3'')); 33.7, 33.8, 34.0 (3*s*, 3 C(2'')); 63.0 (*s*, C(5')); 70.4 (*s*, C(3')); 73.4 (*s*, C(2')); 80.8 (*s*, C(4')); 86.56 (*d* + *s*, ¹*J*(N(9),C(1')) = 12, C(1')); 125.4 (*m*, C(5)); 138.4 (*d*, ¹*J*(N(9),C(8)) = 11, C(8)); 145.2 (dd, ¹*J*(N(1),C(2)) = 8, *J* < 2, C(2)); 148.7 (dd, ¹*J* = 20, *J* = 3, C(4)); 158.9 (*m*, C(6)); 175.3, 175.6, 176.5 (3*s*, 3 C(1'')). ¹⁵N-NMR (41 MHz, CDCl₃, ext. Me¹⁵NO₂, incl. *J*(¹H,¹⁵N)): −210.60 (*mddd*, ²*J*(H–C(8),N(9)) = 8.2, ³*J*(N(3),N(9)) = 3.8, ²*J*(N(7),N(9)) = 2.0, N(9)); −208.35 (br. *m*, N(1)); −163.91 (dd, ²*J*(H–C(2),N(3)) = 13.2, ²*J*(N(9),N(3)) = 3.3, N(3)); −133.25 (dd, ²*J*(H–C(8),N(7)) = 11.6, ²*J*(N(9),N(7)) = 2.0, N(7)). FAB⁺-MS (NBA): 485 (0.5), 484 (4.18), 483 (17.45), 482 (6.16), 481 (1.18) 480 (0.32), 479 (0.11; molecular ions); 345 (2.56), 344 (16.73), 343 (87.80), 342 (2.48), 341 (1.23; Rib2,3,5ib₃ cations); 168 (5.27), 169 (29.32), 143 (0.35), 142 (0.96), 141 (18.92), 140 (6.47), 139 (4.07), 136 (2.12; hypoxanthine cations); 97 (21); 71 (80); 43 (100).

Spectroscopic Characterization of N²-*(2-Methylpropanoyl)-2',3',5'-tris-O-(2-methylpropanoyl)[* N²,1,3,7,9-*¹⁵N]guanosine* (2). ¹H-NMR (400 MHz, CDCl₃, SiMe₄): 1.124, 1.142 (2d(= *t*), *J* = 7, *Me₂*CHCOOC(2') or −C(3')); 1.148, 1.168 (2d, *J* = 7,*Me₂*CHCOOC(3') or −C(2')); 1.225 (*d*, *J* = 7, *Me₂*CHCOOC(5')); 1.303, 1.317 (2d, *J* = 7, *Me₂*CHCON); 2.56, 2.57 (2*sept.*, *J* = 7, Me₂*CH*COOC(2'), Me₂*CH*COOC(3')); 2.63 (*sept.*, *J* = 7,

Me$_2$CHCOOC(5')); 2.73 (*sept.d, J* = 7, ^2J(N–C(2), H–C(2''')) = 0.8, Me$_2$CHCON); 4.50 (*t, J* = 7, H–C(4')); 4.51 (*dd, J* = 14,7, H$_a$–C(5')); 4.62 (*dd, J* = 14,7, H$_b$–C(5')); 5.80 (*dd, J* = 4.9, 3.8, H–C(3')); 5.92 (*dd, J* = 5.1, *J*(H–C(1'),N(9)) = 1.2, H–C(1')); 5.99 (*td, J* = 5.1, ^3J(H–C(2'),N(9)) = 1.7, H–C(2')); 7.6996, 7.6989, 7.6982 (*s + dd + dd + ddd,* ^2J(N(7), H–C(8)) = 11.5, ^2J(N(9),H–C(8)) = 8.5, ^6J(N–C(2),H–C(8)) = 0.8, H–C(8)); 9.03 (*dd + s, J*(*H*N–C(2),N–C(2)) = 92.5, ^3J(*H*N–C(2),N(3)) = 2.1, HN–C(2)); 12.03 (*d + s,* ^1J(H,N(1)) = 91.2, HN(1)). ^{13}C-NMR (101 MHz, CDCl$_3$, SiMe$_4$): 18.7–19.0 (6s, 6 C(3''), 2 C(3''')); 33.7, 33.8, 33.9 (3s, 3 C(2'')); 36.56 (*d + s,* ^2J(N–C(2), C(2'')) = 8, C(2''')); 62.9 (*s,* C(5')); 70.8 (*s,* C(3')); 72.7 (*s,* C(2')); 80.2 (*s,* C(4')); 87.66, 87.68 (*d + s,*^1J(N(9),C(1')) = 12, C(1')); 122.6 (*td,* ^2J(N(9),C(5)) = 4.5, ^2J(N(3),C(5)) = 4.5, ^1J(N(7),C(5)) = 2.1, C(5)); 138.4 (*d,* ^1J(N(9),C(8)) = 11, C(8)); 147.1–147.8 (*m,* C(4), C(2)); 155.3 (*dd,* ^3J(N(9),C(6)) = 9.5, ^3J(N(3),C(6)) = 9.5, ^1J(N(1),C(6)) < 2, C(6)); 175.5, 175.9, 177.7 (3s, 3 C(1'')); 178.725, 178.735 (*dd + s,* ^1J(N–C(2),C(1''')) = 11, ^3J(N(3),C(1''')) = 2.5 C(1''')). ^{15}N-NMR (41 MHz, CDCl$_3$, ext. Me^{15}NO$_2$, incl. *J*(^{1}H,^{15}N)): −247.60 (*ddd,* ^1J(HN–C(2),N–C(2)) = 92.5, ^2J(N(1),N–C(2)) = 16.8, ^2J(N(3),N–C(2) = 6.9, N–C(2)); −228.23 (*dd,* ^1J(HN(1),N(1)) = 91.2, ^2J(N–C(2),N(1)) = 16.8, N(1)); −213.32 (*mddd,* ^2J(H–C(8),N(9)) = 8.6, ^3J(N(3),N(9)) = 3.9, *J*(N,N) = 1.5, N(9)); −199.95 (*ddd,* ^2J(N–C(2),N(3)) = 6.9, ^3J(N(9),N(3)) = 3.9, *J*(N,N) = 1.5, N(3)); −132.83 (*d,* ^2J(H–C(8),N(7)) = 11.6, N(7)). FAB$^+$-MS (NBA): 571 (0.96), 570 (6.11), 569 (22.08), 568 (12.93), 567 (3.76), 566 (0.90), 565 (1.60), 564 (2.28), 563 (0.34; molecular ions); 244 (7.70), 343 (41.96; Rib2,3,5ib$_3$, cations); 229 (0.08), 228 (0.84), 227 (9.55), 226 (5.18), 225 (1.55), 224 (0.49), 223 (0.67), 222 (1.09), 221 (0.18; ib^{2}G cations); 168 (2.30), 169 (22.93), 97 (19), 71 (74), 43 (100).

[N^2,1,3,7,9-^{15}N]Guanosine. A small aliquot of **2** (*ca.* 60 mg) was dissolved in methanolic 0.35N MeONa (*ca.* 3 ml) and stirred overnight at r.t. After neutralization with *Dowex*-H$^+$, TLC (AcOEt/MeOH/H$_2$O 4:1:0.6) showed only small amounts of [N^2,1,3,7,9-^{15}N]guanosine; the major product was N^2-(2-methylpropanoyl)[N^2,1,3,7,9-^{15}N]guanosine. After evaporation of the solvent, 35% aq. NH$_3$ soln. (*ca.* 2 ml) was added and the soln. heated in a sealed vessel at 55° for 4 h. After evaporization of NH$_3$, a semi-prep. HPLC purification of the mixture (flow rate 15 ml min^{-1}; eluant *A* H$_2$O, eluant *B* MeCN/H$_2$O 3:7; 40°; gradient: *B* 0–10% (0–10 min), *B* 10% (10–13 min, *B* 0% (13.1–18 min); detection at 260 nm; t_R 10.4 min) furnished pure, crystalline [N^2,1,3,7,9-^{15}N]guanosine. ^{15}N-NMR (41 MHz, ^{1}H-broadband-decoupled, 0.1M aq. sodium hydrogen phosphate, pH 7.0, internal ^{15}NH$_4$Cl): 51.7 (*s,* N–C(2)); 127.2 (*s,* N(1)); 145.2 (*s,* N(3)); 147.9 (*s,* N(9)); 214.95 (br. *S,* N(7)).

N^2-*(2-Methylpropanoyl)-2',3',5'-tris-O-(2-methylpropanoyl)-O^6-(2''',4''',6'''-triisopropylphenylsulfonyl)-guanosine* (ib^{2}tps^{6}Gib$_3^{2',3',5'}$). To a soln. of 100 mg (177 μmol) of N^2-(2-methylpropanoyl)-2',3',5'-tris-O-(2-methyl-propanoyl)guanosine (ib^{2}Gib$_3^{2',3',5'}$) and 107.2 mg (354 μmol) of 2,4,6-triisopropylbenzenesulfonyl chloride (tps Cl) in dry CH$_2$Cl$_2$ (5 ml), Et$_3$N (98.7 μl, 71.6 mg, 708 μmol) and 4-(dimethylamino)pyridine (*ca.* 1 mg) were added. After 4 h at r.t., the mixture was directly chromatographed (SiO$_2$ (10 g), 10% MeCN/CH$_2$Cl$_2$): 106.3 mg (72%) of ib^{2}tps^{6}Gib$_3^{2',3',5'}$. ^{1}H-NMR (300 MHz, CDCl$_3$, SiMe$_4$): 1.10–1.30 (*m, (Me$_2$CH)$_3$C$_6$H$_2$, 3 Me$_2$CHCOO, Me$_2$CHCON); 2.5–2.7 (*m,* 3 Me$_2$CHCOO); 2.9–3.0 (*m,* Me$_2$CHCON, 4'''-Me$_2$CHC$_6$H$_2$); 4.25 (*quint., J* = 7, 2''',6'''-(Me$_2$CH)$_2$C$_6$H$_2$); 4.44 (*m,* CH$_2$(5'), H–C(4')); 5.8 (*m,* H–C(3'), H–C(2')); 6.13 (*d, J* = 3, H–C(1')); 7.23 (*s,* H–C(3''); H–C(5''')); 7.89 (br. *s,* HN–C(2)); 8.04 (*s,* H–C(8)).

[O^6–^{17}O]Guanosine. In a sealed vessel at −10°, Me$_3$N (123 μl, 80.8 mg, 1.37 mmol) was added to an anh. THF soln. (1 ml) of ib^{2}tps^{6}Gib$_3^{2',3',5'}$ (101.9 mg, 123 μmol) and H$_2$^{17}O (12 μl, 632 μmol; *ca.* 10 atom-% ^{17}O). After 10 min at 0°, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU; 27.4 μl, 28.0 mg, 184 μmol) was added and the mixture stirred for 1 h at r.t. (red soln.). A mixture of 2,4,6-triisopropylbenzenesulfonic acid, ib^2[O^6–^{17}O]Gib$_3^{2',3',5'}$ and partly de-isobutyrylated derivatives resulted (TLC). After evaporation, 35% aq. NH$_3$ soln. (10 ml) was added to the crude product, and the sealed mixture was stirred for 20 h at 55°. After filtration of the suspension, a semi-prep. HPLC purification of the filtrate (flow rate 15 ml min^{-1}, isocratic 3% MeCN/H$_2$O; 40°; detection 270 nm; t_R 6.8 min) furnished 7.4 mg (21%) of [O^6–^{17}O]guanosine. FAB$^+$-MS (glyc + HCl; B = base): 286 (25, [(^{18}O)M + H]$^+$, [(^{17}O)M + 2 H]$^+$, [(^{16}O)M + 3 H]$^+$); 285 (31, (^{18}O)M^+, [(^{17}O)M + H]$^+$, [(^{16}O)M + 2 H]$^+$); 284 (69, [(^{16}O)M + H]$^+$, (^{17}O)M^+); 154 (29, [(^{18}O)B + H]$^+$, [(^{17}O)B + 2 H]$^+$, [(^{16}O)B + 3 H]$^+$); 153 (29, (^{18}O)B$^+$, [(^{17}O)B + H]$^+$, [(^{16}O)B + 2 H]$^+$); 152 (100, [(^{16}O)B + H]$^+$, (^{17}O)B$^+$); all other signals below 15%. ^{17}O-NMR (54 MHz, 0.1M aq. sodium hydrogen phosphate, pH 7.0, external 1,4-dioxane): 245.3 ($w_{1/2}$ ≈ 725 Hz).

6-Dehydroxy-2',3',5'-tris-O-(2-methylpropanoyl)-6-(1''H-1'',2'',4''-triazol-1'' (and 4'')-yl)[1,3,7,9-^{15}N]inosine (**3a** and **3b**, resp.). To a stirred suspension of 1*H*-1,2,4-triazole (1.76 g, 25.5 mmol) in dry MeCN (20 ml), POCl$_3$ (0.55 ml, 6 mmol) was added dropwise at 0°, followed by Et$_3$N (4.1 ml, 29.6 mmol). After 30 min at 0°, a prechromatographed (SiO$_2$, MeCN) and predried (molecular sieves 3 Å) soln. of **1** (825 mg, 1.7 mmol) in MeCN (10 ml) was added and the stirred mixture allowed to warm up to r.t. TLC (8% MeOH/CH$_2$Cl$_2$) showed the complete conversion of the starting material (R_f 0.39) into a double-spot at R_f 0.6 after 45 h. The mixture was diluted with AcOEt (45 ml), concentrated *in vacuo* to half the volume, and extracted with 5% NaHCO$_3$ soln. (2 × 20 ml) and brine. The org. phase was dried (Na$_2$SO$_4$) and evaporated and the crude oil (1.31 g) purified by CC

(SiO$_2$, AcOEt): 725.4 mg (80%) of pure **3a/b** 6:1. ^{1}H-NMR (300 MHz, CDCl$_3$, SiMe$_4$): **3a**: 1.123, 1.150, 1.198, 1.212, 1.245 (5d, $J = 7$, 3 Me$_2$CHCOO); 2.571, 2.631, 2.665 (3 sept., $J = 7$, 3 Me$_2$CHCOO); 4.46 (d, $J = 4$, CH$_2$(5')); 4.51 (dd, $J = 4$, H–C(4')); 5.68 (dd, $J = 1.0$, 5.4, H–C(3')); 5.93 (td, $J = 5.3$, ^3J(H–C(2'),N(9)) = 1.7, H–C(2')); 6.31 (dd, $J = 5.2$, ^2J(H–C(1'),N(9)) < 0.5, H–C(1')); 8.328 (s, H–C(3'')); 8.42 (dd, ^2J(H–C(8),N(9)) = 8.0, ^2J(H–C(8),N(7)) = 12.2, H–C(8)); 8.93 (t, ^2J(H–C(2),N(3)) = ^2J(H–C(2),N(1)) = 15.7, H–C(2)); 9.70 (s, H–C(5'')); **3b**: same as above, except 6.28 (d, $J = 5.2$, H–C(1')); 9.62 (s, H–C(3''), H–C(5'')).

[N^6,1,3,7,9-^{15}N]Adenosine (**4**). To a sat., oily THF soln. (ca. 0.5 ml) of **3a/b** (83.8 mg, 0.16 mmol), a chilled 20% aq. ^{15}NH$_3$ soln. (2 ml) was added rapidly. The biphasic soln. was stirred in a sealed vessel overnight at r.t. and subsequently for 3.5 h at 55–60°. TLC (AcOEt/MeOH/H$_2$O 4:1:0.7) showed the conversion of **3a/b** (R_f 0.8) exclusively into **4** (R_f 0.42) with no traces of inosine (R_f 0.29); however, minor, less polar nucleosidic impurities (R_f 0.58–0.64) did not disappear even after prolonged reaction times. After evaporation, the residual solid was dissolved in warm 10% MeOH/H$_2$O (5 ml of opaque soln.). The crude material was purified by reversed-phase MPLC (20% MeOH/H$_2$O): 52.9 mg (90%) of TLC-pure, crystalline **4**. ^{1}H-NMR (300 MHz, CD$_3$OD, SiMe$_4$): 3.75, 3.89 (2dd, $J = 2.5$, 12.5, CH$_2$(5')); 4.17 (dd, $J = 2.5$, 5.0, H–C(4')); 4.33 (dd, $J = 5$, 2.5, H–C(3')); 4.74 (ddd, $J = 2.5$, 6.3, ^3J(H–C(2'),N(9)) ≈ 4.6, H–C(2')); 5.97 (dd, $J = 6.3$, ^2J(H–C(1'),N(9)) = 1.4, H–C(1')); 8.18 (t, ^2J(H–C(2),N(3)) = ^2J(H–C(2),N(1)) = 14.8, H–C(2)); 8.30 (dd, ^2J(H–C(8),N(9)) = 8.0, ^2J(H–C(8),N(7)) = 11.3, H–C(8)). ^{15}N-NMR (41 MHz, ^{1}H-broadband-decoupled, 0.1M aq. sodium hydrogen phosphate, pH 7.0, internal ^{15}NH$_4$Cl): 57.2 (s, N–C(6)); 148.2 (s, N(9)); 197.7 (s, N(3)); 206.6 (s, N(1)); 210.8 (br. s, N(7)). FAB$^+$-MS (glyc + H$_2$O; B = base): 273 (100, [(^{15}N$_5$)M + H]$^+$); 272 (33, [(^{15}N$_4$)M + H]$^+$, (^{15}N$_5$)M^+); 141 (90, [(^{15}N$_5$)B + H]$^+$); 140 (36, [(^{15}N$_4$)B + H]$^+$, (^{15}N$_5$)B$^+$); 131 (51, [C$_5$H$_7$O$_4$]$^+$).

REFERENCES

[1] G. M. Clore, A. M. Gronenborn, *Science (Washington, D.C.)* **1991**, *252*, 1390.

[2] A. T. Brunger, G. M. Clore, A. M. Gronenborn, R. Saffrich, M. Nilges, *Science (Washington, D.C.)* **1993**, *261*, 328.

[3] E. P. Nikonowicz, A. Sirr, P. Legault, F. M. Jucker, L. M. Baer, A. Pardi, *Nucleic Acids Res.* **1992**, *20*, 4515.

[4] R. T. Batey, M. Inada, E. Kujawinski, J. D. Puglisi, J. R. Williamson, *Nucleic Acids Res.* **1992**, *20*, 4515.

[5] 'Production of Nucleotides and Nucleosides by Fermentation', Eds. S. Teshiba and A. Furuya, Gordon and Breach Science Publishers, New York–London–Paris–Tokyo–Melbourne, 1989.

[6] K. Miyagawa, H. Kimura, K. Nakao, M. Kikuchi, M. Doi, S. Akiyama, K. Nakahama, *BioTechnology* **1986**, *4*, 225.

[7] 'Biochemical Pathways', Ed. G. Michal, Boehringer Mannheim GmbH, Mannheim, 1974.

[8] D. Neuhaus, M. P. Williamson, 'The Nuclear Overhauser Effect in Structural and Conformational Analysis', VCH Publishers, Inc., New York–Weinheim–Cambridge, 1989, Vol. 1, pp. 522.

[9] 'NMR-Spektroskopie von Nichtmetallen: ^{15}N-NMR-Spektroskopie', Eds. S. Berger, S. Braun, and H.-O. Kalinowski, Thieme Verlag, Stuttgart, 1992, Vol. Band 2.

[10] G. E. Hawkes, E. W. Randall, W. E. Hull, *J. Chem. Soc., Perkin Trans.* **1977**, *2*, 1268; V. Markowski, G. R. Sullivan, J. D. Roberts, *J. Am. Chem. Soc.* **1977**, *99*, 714; P. Büchner, W. Maurer, H. Rüterjans, *J. Magn. Reson.* **1978**, *29*, 45; G. Remaud, X. X. Zhou, C. J. Welch, J. Chattopadhyaya, *Tetrahedron* **1986**, *42*, 4057; G. Remaud, J. Kjellberg, H. Bazin, N. G. Johansson, J. Chattopadhyaya, *ibid.* **1986**, *42*, 5073; G. Barbarella, A. Bertoluzza, V. Tugnoili, *Magn. Reson. Chem.* **1987**, *25*, 864; B. Golankiewicz, W. Folkman, H. Rosemeyer, F. Seela, *Nucleic Acids Res.* **1987**, *15*, 9075.

[11] B. L. Gaffney, R. A. Jones, *Tetrahedron Lett.* **1982**, *23*, 2253; N. Bischofberger, *ibid.* **1987**, *28*, 2821; N. Bischofberger, M. D. Matteucci, *J. Am. Chem. Soc.* **1989**, *111*, 3041.

[12] B. F. L. Li, C. B. Reese, P. F. Swann, *Biochemistry* **1987**, *26*, 1086.

[13] Y. Z. Xu, P. F. Swann, *Nucleic Acids Res.* **1990**, *18*, 4061; F. D. Fernandez, Y. Palom, S. Ikuta, E. Pedroso, R. Eritja, *ibid.* **1990**, *18*, 5729.

[14] 'Methoden der organischen Elementar- und Spurenanalyse', Eds. F. Ehrenberger and S. Gorbach, Verlag Chemie, Weinheim, 1973.

[15] 'Molecular Cloning. A Laboratory Manual', Eds. I. Maniatis, E. Fritsch, and J. Sambrock, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.

[16] 'Determinative Bacteriology', 8th edn., Eds. R. E. Buchanan and N. E. Gibbons, The Williams and Wilkins Company, Baltimore, 1975; 'The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria', Eds. M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel, Springer Verlag, Berlin–Heidelberg, 1981.