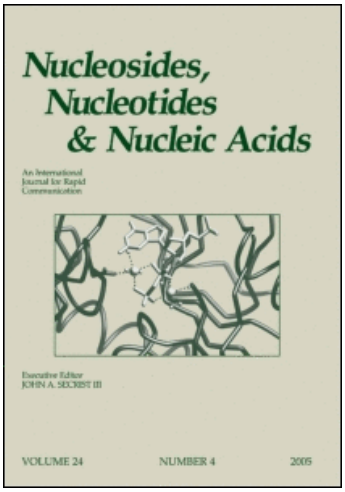


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# Synthesis of 3'-Amino-3'-deoxyguanosine 5'-Triphosphate

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**To cite this Article** Visser, G. M. , Keemink, R. , Schattenkerk, Cecile , Kraal, B. and Van Boom, J. H.(1984) 'Synthesis of 3'-Amino-3'-deoxyguanosine 5'-Triphosphate', Nucleosides, Nucleotides and Nucleic Acids, 3: 3, 277 — 286
**To link to this Article: DOI:** 10.1080/07328318408081264
**URL:** http://dx.doi.org/10.1080/07328318408081264

NUCLEOSIDES & NUCLEOTIDES, 3(3), 277-286 (1984)

# SYNTHESIS OF 3'-AMINO-3'-DEOXYGUANOSINE 5'-TRIPHOSPHATE

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Abstract: Phosphorylation of 2'-O-acetyl-3'-trifluoroacetamido-3'-deoxy-$N^2$-palmitoylguanosine with N-morpholino-O,O-bis(1-benzotriazolyl)phosphate gives a 5'-phosphotriester. Removal of the benzotriazolyl group and addition of pyrophosphoric acid gave, after deblocking all protecting groups, GTP(3'$NH_2$).

Riboguanosine 5'-triphosphate (GTP) binding proteins participate in a large number of biochemical processes, such as hormonal regulation of adenylate cyclase[1], interferon-induced antiviral response[2], assembly of tubulin into microtubules[3], and binding and translocation of aminoacyl-tRNA to ribosomes[4]. For further characterization of the GTP interactions on these proteins a number of GTP derivatives have been described, such as ($^{35}$S)guanosine-5'-O-(3-thiotriphosphate)[1] and the affinity labels 8-azido-guanosine 5'-triphosphate[5] and 5'-p-fluorosulfonylbenzoyl guanosine[6]. For particular proteins, e.g. the peptide chain elongation factor EF-Tu, the stereospecific constraints on the nucleotide cofactor are rather high, and modification of the guanine moiety or at the 5' end causes a large drop in affinity[4]. The partially resolved 3-D structure of EF-Tu·GDP (cf. Fig. 1) indeed reveals hydrogen bond interactions with the base and the 5' phosphates, whereas the 2',3'-hydroxy groups are exposed to the solvent[7]. This led us to the idea of synthesizing GTP(3'$NH_2$) which should allow the introduction of any desired fluores-





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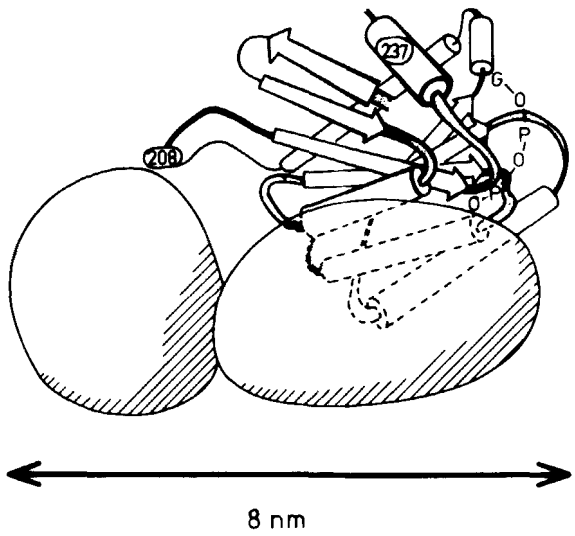
cence or spin label at the 3' position. In the case of EF-Tu, the atom coordinates of the nucleotide at its binding site are well defined and could serve as a reference for the measurement of distant interactions

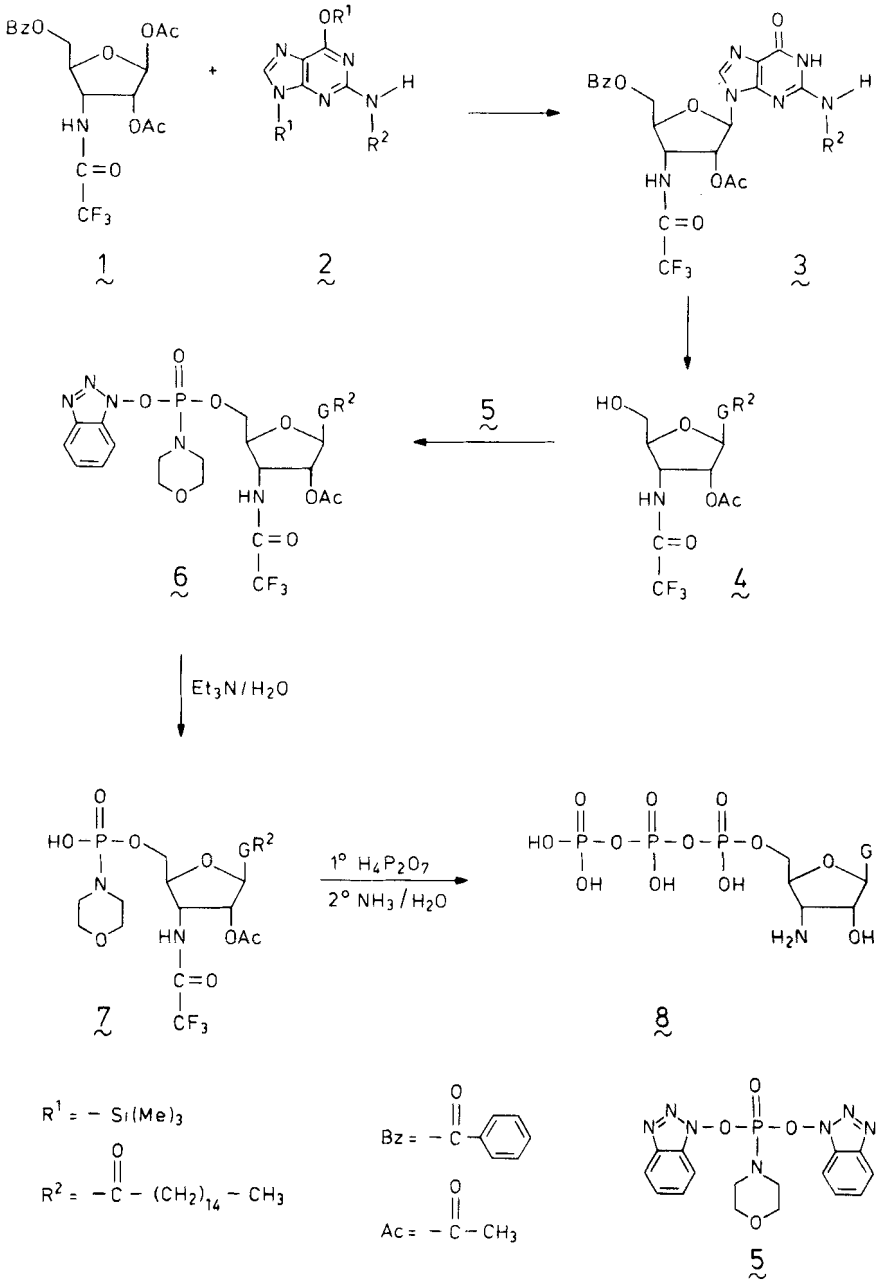
FIG. 1

Three-dimensional model of EF-Tu with details of α-helices (cylinders) and β-strands (arrows) in the nucleotide binding domain. Contact areas with the two tRNA molecules are supposed around residue nrs. 208 and 237. For further details see ref. 7, 9 and 10.




with other ligands such as tRNA with a similarly labelled 3'-amino-3'-deoxyadenosine at its 3' end[8]. In the light of the recent finding that EF-Tu can bind two tRNA molecules simultaneously[9,10], such an approach would be especially useful.

The properly-protected 3'-amino-3'-deoxy-D-ribofuranose derivative 1 (see Scheme) plays a pivot role in our approach to the synthesis of GTP(3'$NH_2$). Key intermediate 1 can be obtained by a multistep process starting either from D-glucose[11,12] or D-xylose[13-15]. On the other hand, Morr et al.[16] obtained intermediate 1 by acetolysis of 3'-amino-3'-deoxy-riboadenosine which could easily be isolated by fermentation from Helminthosporium sp. 125. In our approach, we adopted the recently developed method of Ozols et al.[17] for the preparation of 1. According to this method, the 3-OH group of 1,2-isopropylidene-5-O-benzoyl-β-D-xylofuranose was esterified with trifluoromethanesulfonic acid anhydride followed by substitution of the triflate function with lithium azide. Hydrogenolysis of the 3'-azido function with palladium on charcoal, and protection of the free amino group with trifluoroacetic anhydride[18], afforded the fully-protected 3-amino-3-deoxy-ribose derivative 1. Condensation of 1 with a persilylated $N^2$-acylguanine[19] derivative




Scheme 1

2 ($R^1$=trimethylsilyl; $R^2$=acyl) in 1,2-dichloroethane, and in the presence of the Friedel-Crafts catalyst trimethylsilyl trifluormethanesulfonate[20,21], would afford the fully-protected guanosine derivative 3. However, we found that condensation of 1 with guanine derivative 2, in which $R^2$=acetyl or benzoyl, gave a moderate yield (experimental evidence not given here) of the required derivatives 3 ($R^2$=acetyl or benzoyl). A higher yield of 3 ($R^2$=palmitoyl) was obtained if we subjected the persilylated guanine derivative 2 carrying the lipophilic $N^2$-palmitoyl protecting group[22] to the condensation conditions mentioned above. Careful analysis of the crude reaction mixture by TLC indicated the presence of mainly the N-9 isomer (3) and presumably a small quantity of the N-7 isomer. The fully-protected derivative 3 ($R^2$=palmitoyl) was now converted, by the following steps, into compound 4 having a free 5'-OH. Thus, short treatment of 3 with sodium methoxide, followed by selective protection of the 5'-OH with 4,4'-dimethoxytrityl chloride, afforded, after acetylation of the 2'-OH group with acetic anhydride and acid hydrolysis of the dimethoxytrityl group, compound 4 as a colourless glass.

The introduction of the triphosphate function was accomplished by applying the crystalline and easily accessible bifunctional phosphorylating agent 5[23]. Thus an excess of 5 in dioxane was added to compound 4 in the presence of N-methylimidazole. Work-up of the reaction mixture, after 2 h at 20°C, afforded the phosphotriester derivative 6 as a homogeneous solid in a good yield. Removal of the benzotriazolyl P-O protecting group from 6 to afford crude 7, was accomplished by treatment with triethylamine/water in acetonitrile. Crude 7 thus obtained was treated with the tri-butylammonium salt of pyrophosphoric acid in DMF for 16 h at 20°C. Ammonolysis of the reaction product afforded crude 8 which was purified by anion-exchange column chromatography. The homogeneity and identity of 8 was unambiguously ascertained by $^{31}P$- and $^{1}H$-NMR spectroscopy.

In conclusion, the data presented in this paper show that phosphorylation of the guanosine derivative 4 ($R^2$=palmitoyl) with the bifunctional agent 5 presents a convenient route to the synthesis of GTP(3'-$NH_2$). Preliminary competition experiments between ($^{3}H$)GTP and GTP(3'-$NH_2$) for binding EF-TU revealed an only two-fold decreased affinity for the latter modified ribonucleotide.

## Experimental

General methods and materials: Dioxane, tetrahydrofuran, pyridine and acetonitrile were dried by heating with $CaH_2$ under reflux for 16 h and then distilled. Dimethylformamide was stirred with $CaH_2$ for 16 h and then distilled under reduced pressure. All solvents were stored over molecular sieves 4Å. 1,2-Dichloroethane was washed with concentrated sulfuric acid, water and 10% aqueous $NaHCO_3$ dried over $CaCl_2$ distilled from $P_2O_5$ and stored over molecular sieves 4Å. Hexane, p-xylene and toluene were distilled and stored over sodium. Evaporations were carried out under reduced pressure (15 mm Hg) at 40°C. $^1$H-NMR spectra were measured at 100 MHz using a Jeol JNPS 100 spectrometer or at 300 MHz with a Brüker WM 300 spectrometer, equipped with an Aspect 3000 computer, operating in the Fourier Transform mode. $^{13}$C- and $^{31}$P-NMR spectra were measured with a Jeol JNPS 100 spectrometer equipped with a EC-100 computer, operating in the Fourier Transform mode. Chemical shifts ($\delta$-values) are given in ppm relative to tetramethylsilane ($^1$H-NMR) or tetramethylammonium chloride ($^{13}$C-NMR), and 85% $H_3PO_4$ as an external reference for $^{31}$P-NMR spectroscopy. Compounds were visualized by UV-light, or by spraying wit the appropriate reagents. Thus compounds containing sugar moieties were visualized by spraying with sulfuric acid (20%; v/v) or molybdato phosphoric acid/acetic acid/sulfuric acid (25 g/500 ml/25 ml). Compounds containing aliphatic amino groups were detected by ninhydrine spray reagent (Merck). TLC was performed on Silicagel (DC-fertigfolien F 1500 LS 254, Schleicher & Schüll). Solvent system A: chloroform/methanol (92:8, v/v) unless otherwise stated. Column chromatography was performed on silicagel (Merck, Kieselgel, 230-400 mesh). High performance anion-exchange chromatography was performed with the strong anion-exchange resin Permaphase AAX (Dupont, USA) dry-packed into a stainless-steël column (1 m x 2.1 mm). Gradient elution was affected by building up a linear gradient starting with buffer A (0.005 M $KH_2PO_4$, pH 4.5) and applying 1% of buffer B (0.1 M $KH_2PO_4$, 1.0 M KCl, pH 4.5) per min. A flow of 1 ml/min at a pressure of 8 MP at 20°C was standard.

9'-(2'-O-acetyl-3'-deoxy-3'-trifluoroacetamido-5'-O-benzoyl-β-D-ribofuranosyl)-$N^2$-palmitoylguanosine (3)

$N^2$-Palmitoylguanine (5.5 g, 14.1 mmole) was, after coevaporation with

anhydrous pyridine (3 x 30 ml), refluxed for 7 h with hexamethyldisilazane (HMDS, 25 ml), trimethylchlorosilane (TCS, 0.5 ml) and anhydrous pyridine (5 ml). The excess HMDS and pyridine were removed by coevaporation twice with anhydrous p-xylene (25-50 ml). The solid yellow silyl compound 2 (1.63 g, 3.1 mmole) was dissolved together with compound 1 (1.13 g, 2.6 mmole) in 1,2-dichloroethane (20 ml) and coevaporated twice with anhydrous toluene (2 x 50 ml) to afford an oil. The oil was redissolved in 1,2-dichloroethane (70 ml) and trimethylsilyltriflate (0.85 g, 3.85 mmole) was added. The mixture was gently refluxed in an atmosphere of dry nitrogen. After 6 h, TLC (system A) showed the reaction to be complete. Mainly two products in a ratio of 1:10 could be detected by TLC (A) analysis. The reaction mixture was diluted with chloroform (100 ml) and washed with aqueous sodium hydrogen carbonate (10%, v/v, 25 ml) and water (25 ml). The organic layer was dried ($MgSO_4$) and evaporated to afford a light brown oil. The crude product was dissolved in chloroform and purified on a column (8 x 12 $cm^2$) of Kieselgel (230-400 mesh). Elution of the column with chloroform/methanol (100:0 → 98:2, v/v) afforded 3 as a yellow glass. Yield 1.46 g (73%). Rf(A): 0.36.

$^1$H-NMR data ($CDCl_3/CD_3OD$): 0.8 (t, 3xH, O=C-$(CH_2)_{14}$-$\underline{CH_3}$); 1.25-1.60 [m, 26xH, O=C-$CH_2(\underline{CH_2})_{13}$-$CH_3$]; 2.10 (s, 3xH, $CH_3$-acetyl); 2.38 [t, 2xH, O=C-$\underline{CH_2}$-$(CH_2)_{13}$-$CH_3$]; 4.60 (m, 3xH, $H_4'$, $H_5'$, $H_5''$); 5.50 (t, 1xH, $H_3'$); 5.80 (m, 1xH, $H_2'$); 6.02 (d, 1xH, $H_1'$, $J_{1'-2'}$ = 0.5 Hz); 7.30-7.90 (m, 5xH, arom.); 7.65 (s, 1xH, $H_8$, exo-cyclic base). $^{13}$C-NMR ($CDCl_3$): δ 176.4 s, O=$\underline{C}$-$(CH_2)_{14}$-$CH_3$ ; 170.2 (s, O=$\underline{C}$-$CH_3$); 166.4 (s, 1xO=C, benzoyl); 157.7 (q, O=$\underline{C}$-$CF_3$, $J_{CF}$ = 38.5 Hz); 155.8, 148.3, 148.0, 139.6, 121.5 (s, $C_6$, $C_2$, $C_4$, $C_8$, $C_5$, exo-cyclic base); 133.7, 129.6, 129.2, 128.5 (s, 1 x benzoyl); 115.6 (q, O=C-$\underline{CF_3}$, $J_{CF}$ = 238 Hz); 89.2 (s, $C_1'$); 78.7 (s, $C_4'$); 77.4 (s, $C_2'$); 62.7 (s, $C_5'$); 51.0 (s, $C_3'$); 37.0 (s, O=C-$\underline{CH_2}$-$(CH_2)_{13}$-$CH_3$); 32.1, 29.8, 29.5, 24.9, 22.8 [s, O=C-$CH_2$-$(\underline{CH_2})_{13}$-$CH_3$]; 20.4 (s, $CH_3$, 1 x acetyl); 14.2 (s, O=C-$(CH_2)_{14}$-$\underline{CH_3}$.

9-(2'-O-acetyl-3'-deoxy-3'-trifluoroacetamido-β-D-ribofuranosyl)-$N^2$-palmitoylguanosine (4)

Compound 3 (0.7 g, 0.92 mmole) was dissolved in anhydrous methanol/py-

ridine (8.8 ml, 1/1, v/v) and treated with sodium methoxide (1 M, 2.8 ml). After 10 min, TLC analysis showed the reaction to be complete (System A, 0.36 → 0.15), and the reaction mixture was quenced by the addition of a slight excess of pyridine-HCl salt. The reaction mixture was coevaporated three times with anhydrous pyridine and the precipitated NaCl salts were filtered off over a bed of celite Hyflo supercel. The filtrate was concentrated under reduced pressure to give a yellow oil. To the magnetically stirred solution of the oil (0.5 g ~ 0.81 mmole) in anhydrous pyridine (4 ml) was added dimethoxytrityl chloride (0.34 g, 1.00 mmole). TLC analysis (System A), after 3 h, showed a more lypophilic product [Rf(A): 0.15 → 0.48] and that no starting material was left. Acetic anhydride (0.7 ml, 7.9 mmole) was now added to the reaction mixture. After 16 h at 0-5°C, TLC analysis (System A) showed the reaction to be complete. Water was added (10 ml) and the mixture was concentrated under reduced pressure and coevaporated with toluene (2 x 25 ml), dry ethanol (2 x 25 ml) and chloroform (2 x 25 ml). Short column purification afforded the fully protected compound as a yellow glass. Yield 0.65 g (84%). Rf(A): 0.60. The fully protected compound 4 (0.65 g, 0.68 mmole) was dissolved in 15.4 ml dichloromethane/methanol (3:7, v/v). To the magnetically stirred solution was added 15.4 ml of a stock solution containing 4% benzenesulfonic acid in chloroform/methanol (7:3, v/v). TLC analysis (System A), after 5 min, showed complete removal of the dimethoxytrityl group [Rf(A): 0.60 → 0.25]. The reaction mixture was diluted with chloroform (100 ml) and washed with sodium hydrogen carbonate (10%, v/v, 25 ml) and water (25 ml). The organic layer was dried ($MgSO_4$), concentrated to an oil and triturated with petroleum-ether (40-60°C, 2 x 100 ml). The precipitate was redissolved in chloroform and chromatographed on a Kieselgel column (230-400 mesh). Elution of the column with chloroform/methanol (94:6, v/v) and evaporation of the appropriate fractions afforded pure 4 as a white glass. Yield 0.43 g (71% based on 3). Rf(A): 0.25.

$^1$H-NMR (300 MHz) ($CDCl_3$): $\delta$ = 0.75 (t, 3xH, O=C-$(CH_2)_{14}$-$\underline{CH_3}$); 1.30 (m, 26H, O=C-$CH_2(\underline{CH_2})_{13}CH_3$); 1.70 (t, 3xH, acetyl); 2.52 (t, 2xH, O=C-$\underline{CH_2}$-$(CH_2)_{13}$-$CH_3$); 3.77 (m, 2xH, $H_5'$, $H_5''$); 4.28 (m, 1xH, $H_4'$); 5.05 (t, 1xH, $H_3'$); 5.72 (m, 1xH, $H_2'$); 6.0 (d, 1xH, $H_1'$, $J_{1'-2'}$ = 4 Hz).

Anal. calc. for $C_{30}H_{45}N_6O_7F_3$ (658.72): C, 54.70; H, 6.89; N, 12.76. Found: C, 54.00; H, 6.33; N, 12.90.

9-[2'-O-acetyl-3'-deoxy-3'-trifluoroacetamido-5'-O-phosphoryl-morpholino-(1-benzotriazolyl)-phosphate-β-D-ribofuranosyl]-$N^2$-palmitoyl-guanosine (6)

N-Morpholino-O,O-bis(1-benzotriazolyl)phosphate 5 (5.2 ml of a 0.2 M stock solution in anhydrous dioxane) and 1-methylimidazole (0.42 ml, 4.73 mmole) in anhydrous dioxane (5.2 ml) were added to compound 4 (0.28 g, 0.43 mmole). After stirring for 120 min at 20°C, TLC analysis (System A) showed the reaction to be complete. The reaction mixture was diluted with chloroform (30 ml) and extracted three times with cold triethylammonium bicarbonate (0.9 M, 3 x 6 ml), water (2 x 6 ml), $KH_2PO_4$-solution (0.15 M, 2 x 6 ml, pH 6) and water (1 x 6 ml). The organic layer was dried with petroleum-ether (40-60°C, 100 ml). The precipitate was filtered off and dried in vacuo ($P_2O_5$). Yield of 6, which was isolated as a white precipitate, was 0.32 g (81%). Rf(A): 0.42. $^{31}P\{^1H\}$-NMR ($CDCl_3$): δ 9.36, 8.61 (s, diastereoisomers).

Synthesis of 3'-deoxy-3'-amino-riboguanosine-5'-triphosphate (8)

Compound 6 (0.30 g, 0.32 mmole) was dissolved in acetonitrile (4 ml) and triethylamine (2 ml) and water (1 ml) was added. TLC analysis (System A), after 4 h, showed complete conversion of the starting product into base-line material. To the crude product 7 thus obtained was added dioxane (10 ml) and the mixture was concentrated carefully to a small volume which was evaporated with dry toluene (3 x 15 ml). Anhydrous DMF (4 ml) and the tri-n-butylammonium salt of pyrophosphoric acid in DMF (4 ml, 0.5 M) were added to compound 7, and the solution was kept under the exclusion of moisture at room temperature for a period of 16 h. The mixture was concentrated to a small volume (4 ml) and aqueous ammonia (27 ml, 25%, v/v) was added. After 50 h at room temperature, the mixture was concentrated to a small volume. The resulting mixture was diluted with aqueous HCl (pH 3) (10 ml) and washed with ether (2 x 30 ml). The clear aqueous solution was basified to pH 8 by the addition of triethylamine. The crude product thus obtained was applied on a column (26 x 6 $cm^2$) of DEAE-Sephadex A25 ($HCO_3^-$-form), suspended in 0.05 M triethylammonium bicarbonate (TEAB). The column was eluted with a linear gradient starting from 0.05 M → 1.0 M TEAB in 40 h. The flow rate was 30 ml per h. Fractions of 6 ml were collected and

analysed by anion-exchange HPLC-analysis. Fractions containing the pure product were collected and evaporated under diminished pressure to a small volume and lyophilized. Compound 8 was brought into the sodium-form by passing it through a column (15 x 2 $cm^2$) of Dowex 50W cation-exchange resin (100-200 mesh, sodium-form). The resulting aqueous solution was relyophilized. Yield of 8, which was isolated as a white powder, was 0.100 g (44%).

$^1$H-NMR (300 MHz) ($D_2O$): 4.28 (t, 2xH, $H_5'H_5''$); 4.50 (q, 1xH, $H_4'$); 4.67 (m, 1xH, $H_3'$); 4.88 (q, 1xH, $H_2'$, $J_{1'-2'}$ = 3 Hz, $J_{2'-3'}$ = 7 Hz); 6.0 (d, 1xH, $H_1'$); 8.02 (s, 1xH, $H_8$, exo-cyclic base).

Tetramethylammonium chloride (TMA) was used as an internal reference, δ-values are given relative to tetramethylsilane (TMS) (δTMA - δTMS = 3.18 ppm).

$^{31}P\{^1H\}$-NMR ($D_2O$): At pH 7.3: αP = -11.20 (d, J = 19.5 Hz); βP = -22.3 (dd, J = 19.5 Hz, J = 19.0 Hz); γP = -9.2 (d, J = 19.5 Hz).



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Received May 15, 1984