Purine Nucleosides and Nucleotides Unequivocally in the Syn Conformation: Guanosine and 5’-GMP with 8-tert-Butyl and 8-(α-Hydroxyisopropyl) Substituents

Reynaldo Pless **, Lech Dudycz *, Ryszard Stolarski ** and David Shugar *

Z. Naturforsch. 33 c, 902 — 907 (1978) ; received July 3, 1978

8-Substituted Purines, Purine Nucleosides, Nucleotides, NMR Spectroscopy, Conformation, Syn-Anti Equilibrium, 5’-Nucleotidase

Direct alkylation via a free radical reaction was utilized to prepare 8-tert-butyguanosine-5’-phosphate; this was dephosphorylated with alkaline phosphatase to give the parent nucleoside. The 8-(α-hydroxyisopropyl) derivatives of 5’-GMP and guanosine were prepared in an analogous manner.

The foregoing products, which are necessarily in the syn conformation about the glycosidic bond because of the bulky 8-substituents (van der Waals’ radii 3.5-4 Å), were characterized by elementary analysis, UV spectra and 1H NMR spectroscopy. The changes in chemical shifts of H (1’), H (2’) and H (3’) of each of the foregoing derivatives, relative to those for guanosine and 5’-GMP, were consistent with their being in the syn conformation and, furthermore, pointed to guanosine and 5’-GMP being predominantly anti, in aqueous medium or DMSO. They also demonstrated that, contrary to the prevailing opinion, 8-bromo-5’-GMP and 8-bromoguanosine are not necessarily exclusively in the syn conformation in solution.

While 8-bromoadenosine-5’-phosphate was slowly dephosphorylated by snake venom 5’-nucleotidase, the 8-tert-butyl and 8-(α-hydroxyisopropyl) derivatives of 5’-GMP were fully resistant to this enzyme.

One of the important conformational parameters of purine nucleosides and nucleotides is the orientation of the heterocyclic base about the glycosidic bond. The significance of this parameter has been clearly delineated in a number of enzymatic reactions e.g. binding of dehydrogenase inhibitor analogues of NAD or its components is conditional on these being in the anti conformation [1]. Differences in activation of glycogen phosphorylase by various nucleotides have been ascribed to differences in the glycosyl torsion angles of the latter when bound to the enzyme [2]. Numerous additional illustrations are extant [3 4]. Conversion of purine nucleotide residues to the syn conformation at the polymer level has also been proposed as a source of spontaneous mutations [4], and could well be a factor in some cases of induced mutagenesis.

It is consequently desirable to have available model purine nucleosides fixed in the syn or anti conformation. Typical models of the former have been considered to be 8-substituted nucleosides [1, 5, 6], but serious doubt now exist as to the presumed exclusive syn conformation of 8-bromourine nucleosides [7]. This led to the synthesis of 8-(α-hydroxyisopropyl)adenosine, which is syn in the solid state [8], and would also be expected to be exclusively syn in solution because of the large effective van der Waals’ radius of the hydroxyisopropyl substituent, 3.5 — 4 Å, as compared to about 2 Å for a bromo substituent.

We have elsewhere [9] described the solution conformation, by 1H NMR spectroscopy, of 8-(α-hydroxyisopropyl)-adenosine which, with the aid of several model compounds, was shown to be exclusively syn. We here report on the syntheses, and conformation about the glycosidic bond, of 8-(α-hydroxyisopropyl)-guanosine and its 5’-phosphate, as well as 8-tert-butyguanosine and 8-tert-buty-5’GMP, in which the tert-butyl substituent is even more bulky than hydroxyisopropyl.

Synthetic Procedures

The required 8-substituted derivatives of guanosine were prepared initially as the 5’-monophosphates by the direct addition of the tert-butyl and α-hydroxyisopropyl radials to 5’-GMP.
The synthesis of 8-tert-butyl-5'-GMP was based on a homolytic alkylation reaction. The alkyl radical was generated from pivalyl aldehyde (trimethyl-acetaldehyde) in the presence of potassium persulphate and Fe(NH₄)₂(SO₄)₂. These are conditions which normally lead to formation of reactive acyl radicals from the aldehyde by cleavage of a hydrogen from the carbonyl carbon. However, it was previously noted that the use of isobutylaldehyde for the homolytic acylation of cyclic 3',5'-GMP yielded, in addition to 8-acyl derivatives, appreciable proportions of the 8-isopropyl derivatives [10], most likely as a result of partial decarboxylation of the acyl radical. When we employed pivalyl aldehyde in this reaction, the main product was the alkyl derivative, with only traces (1−2%) of the acyl derivative. This indicated that the tendency of the acyl radical to undergo cleavage to CO and the corresponding alkyl radical increases as one proceeds from a carbonyl bonded primary alkyl, via a secondary alkyl, to a tertiary alkyl. The stabilities of the alkyl radicals increase in the same order. The tert-butyl radical is one of the more stable alkyl radicals, while the isopropyl radical is slightly less stable, so that the presence of the alkyl radicals is a consequence of the conversion of the less stable acyl radical to the more stable alkyl radical.

The reaction is highly sensitive to the presence of oxygen and, in the absence of flushing the reaction mixture with nitrogen, the maximal attainable yield of alkylated product is reduced 4 to 5-fold.

The corresponding 8-tert-butylguanosine was obtained by quantitative dephosphorylation of the nucleotide with E. coli alkaline phosphatase. The nucleoside was then isolated by chromatography on silica gel and crystallized from water. The product gave a satisfactory elemental analysis and was further identified by means of UV and ¹H NMR spectroscopy. This also provided evidence for the identification of the nucleotide.

The synthesis of 8-(a-hydroxyisopropyl)-5'-GMP was based on the di-tert-butyldiaziridine sensitized photoaddition of isopropanol to 5'-GMP, essentially as previously applied by Elad et al. [11]. The starting substance in this case was the tri-n-butylamine salt of 5'-GMP, because of its improved solubility in the aqueous isopropanol solvent employed. The yield of desired product, following purification, was more than 25%. Enzymatic dephosphorylation of the 8-substituted nucleotide yielded the corresponding nucleoside, obtained in crystalline form, identical with that previously reported by Elad et al. [12].

Both 8-tert-butyl- and 8-(a-hydroxyisopropyl)-5'-GMP, as well as 8-(a-hydroxyisopropyl)-5'-AMP, proved fully resistant to Russell viper venom, which is a rich source of 5'-nucleotidase. It is of interest that, under identical conditions, 8-bromo-5'-AMP was slowly dephosphorylated by the snake venom, at a rate about 40-fold lower than the parent AMP. A plausible interpretation of these findings is that the enzyme requires the anti form of the substrate. However, the foregoing observations do not enable us to exclude possible steric effects in the case of the bulky 8-alkyl substituents.

¹H NMR Spectral Analyses

¹H NMR spectral data for the compounds are presented in Tables I and II.

The conformations of the sugar rings, calculated from the proton-proton vicinal coupling constants with the aid of the two-state model of Altona and...
Table II. Chemical shifts (in ppm vs internal TMS) and vicinal proton-proton coupling constants (in Hz) for 8-tert-butylguanosine in DMSO, and comparative values of chemical shifts for guanosine (0.05 m at 22 °C).

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>H(1)</th>
<th>H(2')</th>
<th>H(3')</th>
<th>H(4')</th>
<th>H(5')</th>
<th>J(1',2')</th>
<th>J(2',3')</th>
<th>J(3',4')</th>
<th>J(4',5')</th>
<th>J(5',6')</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-tert-butylguanosine</td>
<td>5.95</td>
<td>5.06</td>
<td>3.87</td>
<td>3.68</td>
<td>3.54</td>
<td>3.29</td>
<td>2.88</td>
<td>2.88</td>
<td>3.19</td>
<td>3.19</td>
</tr>
<tr>
<td>guanosine</td>
<td>5.19</td>
<td>4.41</td>
<td>3.10</td>
<td>3.38</td>
<td>3.22</td>
<td>3.42</td>
<td>3.83</td>
<td>3.38</td>
<td>3.42</td>
<td>3.68</td>
</tr>
</tbody>
</table>

The chemical shifts for the various compounds show characteristic changes in chemical shifts of H(1'), H(2') and H(3') relative to the parent guanosine and 5'-GMP (Tables I and II). These amount to +0.34 ppm, +0.60 ppm and +0.17 ppm for 8-tert-butyl-5'-GMP; +0.75 ppm, +0.55 ppm and +0.16 ppm for 8-(a-hydroxyisopropyl)-5'-GMP; and +0.30 ppm, +0.65 ppm and +0.60 ppm for 8-tert-butylguanosine. These changes in chemical shifts are clearly due to a change in orientation of the base with respect to the glycosidic bond towards a very pronounced preference for, if not exclusively, the form syn. The changes in chemical shifts of H(2') and H(3'), similar for all three compounds, result from the anisotropic influence of the ring N(3) and the ring currents of the pyrimidine moiety in the conformation syn, and are not directly affected by the nature of the 8-substituent. The changes in chemical shifts of H(1'), by contrast, are due to anisotropic interaction with the 8-substituent, since these are close to each other in the conformation syn; and, in accordance with expectations, would depend on the nature of the 8-substituent. The results also indicate that both guanosine and 5'-GMP are preferentially anti, but do not necessarily exclude the presence of a certain proportion in the conformation syn.

Similar changes in chemical shifts of the sugar protons have been observed for other derivatives of guanosine with bulky 8-substituents, e.g. 8-bromo- and 8-dimethylamino-guanosines [14]. These exhibit the characteristic strong deshielding of H(2'), by 0.51 ppm and 0.57 ppm, respectively, relative to guanosine, and a small change in the chemical shift of H(1'), which depends on the nature of the 8-substituent. These effects testify to a constrained preference of the conformation syn by the 8-substituents. It may be assumed, as in the case of the corresponding adenosine derivatives [9], that the magnitude of the change in chemical shift of H(2') reflects the changes in populations of the syn and anti conformers (in equilibrium with each other), the glycoside torsion angles remaining unchanged.
The change in chemical shifts of H(2') for 8-bromoguanosine (0.51 ppm) is 0.14 ppm lower than that for the fully syn 8-tert-butylguanosine (0.65 ppm), suggesting that 8-bromoguanosine is partly in the form anti. For 8-dimethylaminoguanosine the change in chemical shift of H(2') relative to guanosine is 0.57 ppm, hence closer to that for 8-tert-butylguanosine, indicative of a higher population of the form syn, but still not to the extent of 100%.

The changes in chemical shifts of H(2') for 8-tert-butyl-5'-GMP and 8-(α-hydroxyisopropyl)-5'-GMP, relative to 5'-GMP, differ by 0.1 ppm. Since the 8-substituents in these instances are comparable in size, and sufficiently bulky to ensure a 100% syn population, the unequal changes in chemical shifts must be due to differences in the glycosidic torsion angles. An analogous situation probably prevails for 8-bromo- and 8-dimethylaminoguanosines.

Under conditions of chemical exchange involving the syn = anti equilibrium, the chemical shifts of H(2') would be a linear function of the relative populations. The change in chemical shift of H(2') for a change in conformation from anti to syn should be about 0.65 ppm (this is the difference in shift between the fully syn 8-tert-butylguanosine and the preferentially anti guanosine). From the chemical shift of H(2') for 8-bromoguanosine, which is 0.14 ppm less than that for 8-tert-butylguanosine, it may be inferred that 8-bromoguanosine should have a population anti of about 15%. Bearing in mind possible differences in glycosidic torsion angles, it is more likely that the anti population of 8-bromo-guanosine is somewhat lower than 15%. In addition there is an observed dependence of the anti population of 8-substituted guanosines on the bulkiness of the substituent which constrains the conformation to syn: the greater the bulkiness of the substituent, the greater the population of the syn conformer.

Experimental

Guanosine-5'-phosphate was obtained from Calbiochem (Los Angeles, Calif.) and Reanal (Budapest). Trimethyl acetaldehyde was a product of K & K Laboratories (California, U.S.A.). Di-tert-butylperoxide was purchased from Merck-Schuchardt (Hohenbrunn, Munchen, GFR). Desalting was carried out with Sigma (St. Louis, Mo., U.S.A.) charcoal and celite 501 from Vulcascot.

Melting points (uncorr.) were measured on a Boetius microscope hot stage. Elementary analyses were run on a Perkin-Elmer 240 instrument. UV spectra were obtained with a Zeiss (Jena, GDR) VSU-2P manual spectrophotometer, and a Specord (Zeiss) UV-VIS recording instrument.

1H NMR spectra were recorded on a Bruker-90 instrument, operating in the Fourier transform mode, at room temperature (22°C), using 0.05 M solutions in 2H2O (Merck, Darmstadt, GFR, 99.7 mol % 2H) adjusted to pD 8, with DDS as internal standard; or in DMSO-d6 (Merck, 99.5 mol % 2H), with TMS as internal standard.

E. coli alkaline phosphatase was a product of Sigma (St. Louis, Mo., U.S.A.), while Russell viper venom was used as a source of 5'-nucleotidase.

8-tert-butylguanosine-5'-phosphate. To a solution of 2.08 g (4.7 mmol) of 5'-GMP·Na2·2 H2O in 160 ml of 50% aqueous acetic acid at 10°C was added 25 ml of 3 N aqueous H2SO4 and 19 ml (210 mmol) of pivalyl aldehyde. The solution was purged with nitrogen, and solutions of 14.5 g (37 mmol) of Fe(NH4)2(SO4)2·6 H2O in 60 ml H2O, and 10 g (37 mmol) of K2S2O5 in 100 ml H2O, were added simultaneously, dropwise, with constant stirring over the course of 1 h, the solution being maintained at 10°–15°C. The reaction mixture was stirred for an additional hour at room temperature, diluted by addition of 300 ml H2O, and percolated through a mixture of 60 g charcoal and 30 g Celite. The charcoal bed was washed with 2.51 of H2O, followed by 2.21 of EtOH-H2O-conc. NH4OH (10:10:1, v/v). The ammoniacal alcohol eluate was concentrated to 25 ml under pressure and applied to a 80×2.8 cm column of 200–400 mesh AG 50 W-X 2 (H+). The column was eluted with H2O at a flow rate of 220 ml/h. Material with absorption maxima at 279 nm and 330 nm (presumably 8-pivalyl-5'GMP) eluted at 370 ml, unreacted GMP at 640–2100 ml, and the desired product from 2100–3100 ml (10,000 OD255 units, 0.62 mmol). 3,000 OD255 units of the eluate, concentrated to small volume under reduced pressure, were deposited as a band on Whatman 3 MM paper, which was developed with n-BuOH-H2O-CH3COOH (5:3:2, v/v). The band with Rf = 0.4 was eluted with water. Aqueous ammonia was added and the
solution concentrated to a gum. The residue was taken up in 0.5 ml water, and the product precipitated by addition of ethanol, and dried over P$_2$O$_5$ under vacuum. Yield, 92 mg (14.0%). UV absorption, in aqueous medium: pH 2: $\lambda_{\text{max}}$ 263.5 nm ($\epsilon_{\text{max}}$ 15.1x10$^3$), $\lambda_{\text{min}}$ 230 nm ($\epsilon_{\text{min}}$ 2.7x10$^3$); pH 7: $\lambda_{\text{max}}$ 255.5 nm ($\epsilon_{\text{max}}$ 16.2x10$^3$), $\lambda_{\text{min}}$ 223 nm ($\epsilon_{\text{min}}$ 3.9x10$^3$); pH 12: $\lambda_{\text{max}}$ 260.5 nm ($\epsilon_{\text{max}}$ 14.7x10$^3$); $\lambda_{\text{min}}$ 229 nm ($\epsilon_{\text{min}}$ 4.7x10$^3$).

8-tert-butylguanosine. 7,000 OD$_{255}$ units of 8-tert-butylguanosine-3'-phosphate (column eluate, without further purification) were brought to pH 7: $\lambda_{\text{max}}$ 255.5 nm ($\epsilon_{\text{max}}$ 15.1x10$^3$), $\lambda_{\text{min}}$ 230 nm ($\epsilon_{\text{min}}$ 2.7x10$^3$); pH 12: $\lambda_{\text{max}}$ 260.5 nm ($\epsilon_{\text{max}}$ 14.7x10$^3$); $\lambda_{\text{min}}$ 229 nm ($\epsilon_{\text{min}}$ 4.7x10$^3$).

8-(a-Hydroxyisopropyl)guanosine. To 64 mg of 8-(a-hydroxyisopropyl)guanosine-5'-phosphate in 3 ml of 0.1 M Tris-HCl buffer pH 8.0, containing 0.002 M MgCl$_2$, was added 14 units of E. coli alkaline phosphatase and the mixture incubated at 37°C for 24 h. The digest was then subjected to chromatography on two 20x20 cm silica gel thick layer plates with chloroform-methanol (7:3, v/v). The band at $R_f = 0.4$ was eluted with chloroform-methanol (3:2, v/v), the eluate brought to dryness and the residue crystallized from 1.5 ml hot water. The crystals were washed with ice-cold water and dried under vacuum over P$_2$O$_5$, yield 25 mg, m. p. 212 – 214°C. Concentration of the mother liquors to 0.5 ml yielded an additional 3 mg (total 28 mg, 54%), m. p. 214 – 215°C (Elad et al. give 211 – 213°C). Elem. analysis: C, 43.56%; H, 5.56%; N, 19.38%; calcd. for C$_{14}$H$_{21}$N$_5$O$_6$·H$_2$O: C, 43.58%; H, 5.59%; N, 19.55%.

8-(a-Hydroxyisopropyl)guanosine-5'-phosphate. To a solution of 443 mg (1.0 mmol) of 5'-GMP·Na$_2$·H$_2$O, 1.2 mmol HCl and 2.1 mmol (0.50 ml) of tri-n-butylamine in 60 ml of H$_2$O–2-propanol (5:4, v/v) was added 2 ml of tert-buty1 peroxide. The mixture was irradiated in a photochemical reactor with a 700-watt medium pressure mercury lamp, the radiation from which was filtered through a 5-mm layer of 0.015 M adenine in dimethylformamide, which cuts off at about 270 nm. After 28 h the reaction mixture was applied to a 4.5x2.5 cm column of 200 – 400 mesh Dowex 1 X-4 (formate form). The column was washed with 300 ml water, 300 ml 0.5 M aqueous formic acid, 100 ml of 0.5 M formic acid in 50% aqueous ethanol, and finally with 120 ml of 4 M aqueous formic acid. The eluate from the final wash was brought to dryness under reduced pressure and the residue taken up in aqueous ammonia. The solution was concentrated to small volume, and methanol added to give a precipitate which was subjected to chromatography on Whatman 3 MM paper with n-propanol–conc. NH$_4$OH – water (20:20:3, v/v). The band with $R_f = 0.54$ was eluted with water and concentrated under reduced pressure to a glass, 123 mg (27%) after drying under vacuum over P$_2$O$_5$.

8-(a-Hydroxyisopropyl)guanosine. To 443 mg of 8-(a-hydroxyisopropyl)guanosine-5'-phosphate in 3 ml of 0.1 M Tris-HCl buffer pH 8.0, containing 0.002 M MgCl$_2$, was added 14 units of E. coli alkaline phosphatase and the mixture incubated at 37°C for 24 h. The digest was then subjected to chromatography on two 20x20 cm silica gel thick layer plates with chloroform-methanol (7:3, v/v). The band at $R_f = 0.4$ was eluted with chloroform-methanol (3:2, v/v), the eluate brought to dryness and the residue crystallized from 1.5 ml hot water. The crystals were washed with ice-cold water and dried under vacuum over P$_2$O$_5$, yield 25 mg, m. p. 212 – 214°C. Concentration of the mother liquors to 0.5 ml yielded an additional 3 mg (total 28 mg, 54%), m. p. 214 – 215°C (Elad et al. give 211 – 213°C). Elem. analysis: C, 43.56%; H, 5.56%; N, 19.38%; calcd. for C$_{14}$H$_{21}$N$_5$O$_6$·H$_2$O: C, 43.58%; H, 5.59%; N, 19.55%.

One of us (R. P.) would like to express his thanks to the Polish Academy of Sciences for a fellowship. This investigation was supported by the Polish National Cancer Research Program (PR-6/1701).