Conformation about the Glycosidic Bond and Susceptibility to 5′-Nucleotidase of 8-Substituted Analogues of 5′-GMP

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Susceptibilities to snake venom 5′-nucleotidase (EC 3.1.3.5) have been evaluated for several 8-substituted analogues of 5′-GMP with varying populations of *syn*/*anti* conformations about the glycosidic bond. Improved syntheses of some of these are described, including direct chlorination of 5′-GMP to give 8-chloro-5′-GMP, a procedure which should be applicable to other purine nucleotides. The conformations of the various analogues were determined by means of ^{1}H NMR spectroscopy, with particular emphasis on the glycosidic bond conformations. All the 8-substituted derivatives of 5′-GMP were relatively poor substrates of 5′-nucleotidase. This was shown to result largely from steric effects and the nature of the 8-substituent, and consistent with a requirement for the *anti* conformation. Although ribose-5-phosphate was not a substrate, it was a weak inhibitor, and its inhibitory properties account in part for the weak inhibitory properties of the 8-substituted 5′-GMP, and other, analogues. Attention is drawn to the hitherto largely neglected differences in properties of 5′-nucleotidases from different sources and their relevance to the present findings.

The ubiquitous enzyme 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5), which catalyzes the dephosphorylation of nucleoside-5′-phosphates, was until recently considered relatively non-specific with reference to the heterocyclic base, which appeared to affect only the rate of dephosphorylation. This concept must now be modified in the light of the isolation from blood cells of a 5′-NPase with a high preference for pyrimidine nucleotides [1], and another such enzyme, widely distributed in mammalian tissues, which appears to be specific for orotidine-5′-phosphate, and has been denoted as 5′-OMPase [2]. Furthermore, 5′-NPase, widely profited from as a plasma membrane marker, has been found to be associated with immunodeficiency diseases [3], and its level in physiological fluids is considered to be a promising diagnostic test for hepatic involvement in malignant lymphoma patients [4].

Various studies have been carried out on the mechanism of action of 5′-nucleotidase, including the stereochemical course of hydrolysis [5], and the conformation of the heterocyclic base about the glycosidic bond [6–8]. In the case of purine nucleotides, it has been proposed, with the aid of 5′-AMP analogues fixed in the *anti* conformation, that snake venom 5′-NPase requires the conformation *anti*, and the *gauche-trans* conformation of the exocyclic group [7, 8]. This aspect assumes additional significance when considered in relation to the newly reported 5′-OMPase, since 5′-OMP is necessarily fixed in the *syn* conformation as a result of severe steric hindrance between the carboxyl group at C(6) and the ribose moiety [9].

In the purine nucleoside series, which normally exhibit a dynamic equilibrium between the *syn* and *anti* conformations, this equilibrium may be shifted in the direction *syn* by insertion of a bulky substituent at C(8), and we have shown elsewhere the application of ^{1}H and ^{13}C NMR spectroscopy to determination of the populations *syn* and *anti* as a

Abbreviations: 5′-NPase, 5′-nucleotide phosphohydrolase or 5′-nucleotidase (EC 3.1.3.5); 5′-OMPase, 5′-nucleotidase specific for orotidine-5′-phosphate; acycloG, acycloguanosine or 9-(2-hydroxyethoxymethyl)guanine; acyclo-GMP, acycloG monophosphate; DSS, sodium 2,2-dimethyl-2-silapentane sulfonate; TMS, tetramethylsilane.

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function of the van der Waals' radius of the C(8) substituent [10].

In the present study, we have prepared a series of 8-substituted 5′-GMP analogues, and examined their conformations in solution, as well as their susceptibilities to snake venom 5′-NPase. Additional interest attaches to such studies when it is noted that 8-aminoguanosine is a potent inhibitor of purine nucleoside phosphorylase [11], and that 8-parasubstituted benzylthio derivatives of 5′-AMP and 5′-IMP are inhibitors of IMP dehydrogenase [12].

Materials and Methods

Guanosine and 8-bromoguanosine were from Sigma (St. Louis, MO., USA), 5′-GMP from Calbiochem (Lausanne, Switzerland), and ribose-5-phosphate from Reanal (Budapest, Hungary). AcycloG was a kind gift of Dr. G. B. Elion of Burroughs-Wellcome, and was converted to acycloGMP by the method of Yoshikawa *et al.* [13].

Darco G60 activated charcoal and Celite 501, used for desalting of nucleosides, were from Serva (Heidelberg, GFR) and POCH (Gliwice, Poland) respectively. Commercial $POCl_3$ was distilled under anhydrous conditions prior to use. Metachloroperbenzoic acid, 85% (Ralph Emmanuel, Wembley, UK) was purified before use by washing with pH 7.5 phosphate buffer, water, and drying under vacuum over P_2O_5. Dimethylformamide was purified by distillation with water and benzene, followed by distillation under reduced pressure, and then dried over 4 Å molecular sieves. Trimethylphosphate was purified by distillation under reduced pressure.

Dowex resins were from BioRad (Richmond, CA., USA); Sephadex from Pharmacia (Uppsala, Sweden); Tris buffer, analytical grade, from Fluka (Switzerland); ammonium molybdenate, analytical grade, from POCH (Gliwice, Poland); and L(+)-ascorbic acid, analytical grade, from Merck (Darmstadt, GFR). Other reagents were all analytical grade.

Melting points (uncorrected) were measured on a Boetius (Leipzig, GDR) microscope hot stage.

Thin-layer chromatography made use of Merck silica gel 60 F_{254}, and cellulose F_{254}, plates, with the following solvent systems: (A) isopropanol-water-25% NH_4OH (7:1:1, v/v); (B) isopropanol-water-25% NH_4OH (4:4:1); (C) isopropanol-water-HCOOH (4:4:1).

Column chromatography was with an LKB 2070 Ultrorac II fraction collector with automatic recording at 254 nm.

Enzymatic reactions were carried out with Sigma 5′-nucleotidase from *Crotalus adamanteus* in 0.1 M Tris-HCl buffer pH 7.5, as described by Sulkowski *et al.* [14]. An enzyme unit was that which hydrolyzed 1 µmol 5′-AMP per min at 37 °C at pH 9. Inorganic phosphate was determined according to Ames [15], the absorption of the molybdenate complex being measured with a Zeiss (Jena, GDR) VSU2P instrument. All glassware, including micropipettes, was scrupulously cleaned without the use of detergents. Under the assay conditions employed, 2′(3′)-AMP was not detectably hydrolyzed, thus excluding the presence of non-specific phosphatase.

UV absorption spectra were recorded on a Zeiss Specord UV-VIS spectrophotometer.

^1H NMR spectra were run on a JEOL-JNM-4M 100 instrument with 0.2 M solutions of 5′-GMP and its analogues in ^2H_2O at pH 7, and on guanosine and its analogues in $(C^2H_3)SO$, all at 40 °C. Chemical shifts were measured *vs.* TMS in $(C^2H_3)_2SO$, to an accuracy of 0.01 ppm. Coupling constants are accurate to 0.2 Hz.

Chemical syntheses

The various 8-substituted nucleosides and nucleotides were prepared according to published procedures, or modifications of these, as described below. The following points are pertinent. The previously reported method for the synthesis of 8-(α-hydroxyisopropyl)guanosine and its 5′-phosphate [6] has been considerably improved by appropriate modification of the irradiation conditions. The recent method described by Ryu and MacCoss [16] for chlorination of purine nucleosides has been shown to be applicable, with suitable modifications, for the direct chlorination of nucleotides, such as 5′-GMP, following which the nucleoside may be obtained by enzymatic dephosphorylation (see below). Furthermore, the difficulty in purification of 8-aminoguanosine was circumvented by the synthesis of 8-amino-5′-GMP, followed by enzymatic dephosphorylation of the latter. In the case of 8-methylguanosine, phosphorylation was

carried out by two methods, enzymatic and chemical, the products in both cases being identical.

The purity of all compounds was checked by chromatography, UV spectroscopy, and chemical and/or enzymatic interconversion of nucleosides and nucleotides. However, the final criteria for verification of structures were furnished by the ^{1}H NMR spectra (see below).

8-Methylguanosine was prepared from guanosine as described by Kawazoe *et al.* [17]. It was desalted on actived charcoal with Celite [18] and crystallized from water. Although contaminated with traces of guanosine, it was employed for phosphorylation as such. A small sample, 100 mg, was further purified by elution with a formic acid gradient on a 200–400 mesh Dowex 1 × 4 ($HCOO^-$) column, and recrystallized from water, m.p. 188–190 °C, as compared to a literature value of 185 °C [17]. On silica gel with solvent system A, $R_f = 0.48$ as compared to 0.32 for guanosine.

8-Methyl-5′-GMP was obtained by the following two procedures: (a) Enzymatic phosphorylation. This was carried out by phosphorylation of 8-methylguanosine with the wheat shoot phosphotransferase system as described by Giziewicz and Shugar [19], the product being isolated on a Dowex 1×4 ($HCOO^-$) column, free of guanosine and 5′-GMP; (b) Chemical phosphorylation. This was performed as described by Yoshikawa *et al.* [13]. The reaction mixture was then neutralized with saturated $NaHCO_3$ and deposited on a column of DEAE Sephadex A 25 (HCO_3^-), which was washed with water. Elution was with a linear gradient of 0–0.6 M NH_4HCO_3. Traces of 5′-GMP were eluted at 0.2 M solvent, followed by 8-methyl-5′-GMP. The pooled eluates of the latter were freed of solvent by evaporation under reduced pressure with water, aqueous triethylamine and ethanol, and ethanol. The product was converted to the ammonium salt on a column of Dowex 50 W×8 (NH_4^+), precipitated with acetone and dried under vacuum over P_2O_5 (yield 33%). It was chromatographically homogeneous, with $R_f = 0.62$ and 0.52 with solvents B and C on cellulose plates. UV absorption: pH 2, λ_{max} 260 nm (ε_{max} 13.5×10^3); pH 7, λ_{max} 254 nm (ε_{max} 13.5×10^3); pH 12, λ_{max} 261 nm (ε_{max} 13.7×10^3). On treatment with 5′-nucleotidase, it was slowly converted to 8-methylguanosine.

8-Bromo-5′-GMP was prepared by bromination of 5′-GMP as reported by Ikehara *et al.* [18], with some modifications. Bromination was conducted in 1 M acetate buffer pH 4 for 2 h, with addition of bromine in two portions. Following removal of inorganic salts on a column of charcoal with Celite, the product was separated from unreacted 5′-GMP on a column of DEAE Sephadex A25 (HCO_3^-), converted to the sodium salt on a column of Dowex 50 W×8, precipitated with acetone and dried under vacuum over P_2O_5. The white product was chromatographically homogeneous (yield 37%), with $R_f = 0.62$ and 0.51 with solvents B and C on cellulose plates. UV absorption: pH 2, λ_{max} 264 nm (ε_{max} 15.6×10^3); pH 7, λ_{max} 264 nm (ε_{max} 15.5×10^3); pH 12, λ_{max} 272 nm (ε_{max} 13.9×10^3). On treatment with an excess of 5′-nucleotidase, it was slowly converted to 8-bromoguanosine.

8-Chloroguanosine. This was prepared by treatment of guanosine with anhydrous HCl in dimethylformamide (DMF), essentially according to Ryu and MacCoss [16], slightly modified, as follows: *m*-chloroperbenzoic acid was added as a solution in DMF (0.013 mol in 5 ml DMF) dropwise for one hour, and mixing continued for 30 min at room temperature. The clear solution was brought to a brownish oil. The latter was treated with water, the *m*-chlorobenzoic acid filtered off, the filtrate diluted with water and brought to about pH 6 with dilute NaOH, and extracted several times with ethyl ether. The aqueous phase was then brought to dryness and the product crystallized from water. The cream-colored crystals were chromatographically homogeneous on cellulose plates with solvents B ($R_f = 0.69$) and C ($R_f = 0.73$) and on silica gel with the solvent system acetonitrile-0.1 N NH_4Cl (7:3, v/v, $R_f = 0.78$). Yield 36%. Recrystallization gave white needles, which turned yellow at 200 °C, but did not melt at 275 °C.

8-Chloro-5′-GMP was obtained (a) by phosphorylation of 8-chloroguanosine as described above for 8-methylguanosine, and (b) by direct chlorination of 5′-GMP as follows: To a solution of 1.46 g (4 mmol) of carefully dried 5′-GMP in 80 ml anhydrous DMF was added 2 ml of DMF saturated with HCl (4.4 mmol). To this solution, with constant stirring at room temperature, was added, dropwise, over a period of 1½ h, a solution of 700 mg (4 mmol) of *m*-chloroperbenzoic acid in 10 ml DMF. The mixture was stirred for an additional hour, and then brought to an oil under reduced pressure (at a temperature not exceeding 35 °C). This was

neutralized with dilute NaOH, the resulting precipitate filtered off, and the filtrate extracted three times with ether. The aqueous phase was brought to pH 7, filtered, and the filtrate brought to dryness below 35 °C. The product was dissolved in 50 ml water and deposited on a 15 × 3.5 cm column of DEAE Sephadex A25 (HCO_3^-). The column was washed with 1 l water, and elution then conducted with a linear gradient of 0–0.6 M NH_4HCO_3. The pooled fractions containing 8-chloro-5′-GMP (eluted at 0.2 M NH_4HCO_3) were brought to dryness, the product converted to the potassium salt on a column of Dowex 50 W × 12 (K^+), and precipitated with ethanol-acetone, to yield, following drying over P_2O_5, 980 mg (52%) of an amorphous white powder, chromatographically homogeneous with solvents B and C (R_f = 0.64 and 0.52, as compared to 0.50 and 0.48 for 5′-GMP). UV absorption: pH 2, λ_{max} 261 nm (ε_{max} 15.6 × 10^3); pH 7, λ_{max} 261 nm (ε_{max} 15.3 × 10^3); pH 12, λ_{max} 271 nm (ε_{max} 14.1 × 10^3).

8-Aminoguanosine and 8-amino-5′-GMP were synthesized according to published procedures [20], starting from 8-bromoguanosine. The nucleotide was readily converted to the nucleoside by 5′-nucleotidase.

8-(α-Hydroxyisopropyl)-5′-GMP was prepared essentially as described by Pless *et al.* [6], with some modifications. A closed Pyrex UV-reactor was employed, so that irradiation was limited to wavelengths to the red of 300 nm, and the irradiated solution was kept free of oxygen by purging with argon. This led to an appreciable reduction in formation of tarry side-products, thus facilitating isolation, and increasing the yield, of the desired product. The latter was isolated by chromatography on a column of DEAE Sephadex A25 (HCO_3^-), converted to the sodium salt with Dowex 50 W × 12 (Na^+), and precipitated by addition of ethanol as a white powder (yield 61% following drying over P_2O_5). It was further purified by chromatography on DEAE Sephadex A25 (HCO_3^-), and was chromatographically homogeneous on cellulose plates with solvents B and C (R_f = 0.68 and 0.56). UV absorption: pH 2, λ_{max} 261 nm (ε_{max} 13.9 × 10^3); pH 7, λ_{max} 259 nm (ε_{max} 14.8 × 10^3); pH 12, λ_{max} 261 nm (ε_{max} 13.9 × 10^3).

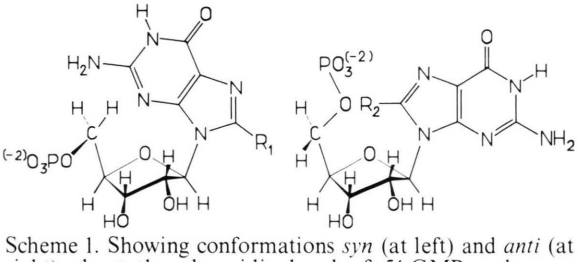
8-(α-Hydroxyisopropyl)guanosine was obtained by quantitative dephosphorylation of the 5′-phosphate with alkaline phosphatase, and crystallized from water as described by Pless *et al.* [6].

Results and Discussion

Conformational features

Table I exhibits the proton chemical shifts and the coupling constants for the sugar protons of 5′-GMP, its 8-substituted analogues, and the corresponding parent nucleosides. The resulting calculated conformations of the bases about the glycosidic bonds, and of the sugar rings and exocyclic groups, are listed in Table II.

Conformation about glycosidic bond. It was previously shown [10, 21] that introduction of a bulky substituent at C(8) of purine nucleosides and nucleotides leads to characteristic changes in chemical shifts of the sugar protons, particularly H(2′) and, to a lesser extent, H(1′) (see Table I). These changes are due to a shift in the dynamic *syn* ⇀ *anti* equilibrium, which exists for the parent nucleosides and nucleotides, in the direction *syn* for the 8-substituted analogues because of unfavourable steric hindrance between the 8-substituents and the sugar ring in the conformation *anti* (see Scheme 1). With an increase in the van der Waals' radius of the 8-substituent, there is an increase in the value of the chemical shift of H(2′), from 4.72 ppm in 5′-GMP and 4.40 ppm in guanosine to 5.24 ppm and 4.95 ppm for the 8-(α-hydroxyisopropyl) analogues, respectively. The behaviour of the chemical shifts of H(1′) is not as clear-cut, and depends to a large extent on the nature of the 8-substituent. This is readily explicable by the fact that, with a marked preference for the conformation *syn*, the C(8) substituent is located in the vicinity of H(1′) and therefore affects its chemical shift *via* inductive and electrostatic effects, dependent on the type of substituent. Modifications of chemical shifts of H(2′) are due to similar effects originating from the pyri-



Scheme 1. Showing conformations *syn* (at left) and *anti* (at right) about the glycosidic bond of 5′-GMP and some 8-substituted analogues: R_1 and R_2 = H, or Cl, or Br, or NH_2, or CH_3. With $R_1 = COH(CH_3)_2$, only the *syn* conformation is possible.

Table I. Chemical shifts of the sugar protons (in ppm *vs.* internal DSS) of 5′-GMP and its 8-substituted analogues in ^2H_2O, and of guanosine and its 8-substituted analogues (in ppm *vs.* internal TMS) in $(CH_3)_2SO$, and the corresponding proton-proton vicinal coupling constants (in Hz).

Nucleotide or Nucleoside	H(1′)	H(2′)	H(3′)	H(4′)	H(5′)[a]	*J*(1′,2′)	*J*(2′,3′)	*J*(3′,4′)	*J*(4′,5′)[b]
5′-GMP	5.90	4.72	4.49	4.32	4.02	5.3	5.1	3.9	3.5
8-NH_2-5′-GMP	5.88	4.78	4.48	4.31	4.11	7.3	5.8	2.5	2.8
8-CH_3-5′-GMP	5.83	5.10	4.53	4.26	4.15	6.1	5.5	3.5	4.4
8-Cl-5′-GMP	5.94	5.16	4.58	4.25	4.13	5.9	5.6	3.8	5.2
8-Br-5′-GMP	5.94	5.22	4.60	4.24	4.15	6.0	5.3	3.2	4.8
8-(α-Hydroxyisopropyl)-5′-GMP	6.64	5.24	4.68	4.20[c]	4.20[c]	5.3	5.9	4.0	[c]
Guanosine	5.70	4.40	4.09	3.89	3.57	5.8	4.9	3.4	3.6
8-NH_2-guanosine	5.76	4.59	4.14	3.91	3.66	7.1	5.6	2.2	3.0
8-CH_3-guanosine	5.68	4.70	4.08	3.85	3.59	6.7	5.7	3.0	3.9
8-Cl-guanosine	5.71	4.93	4.13	3.86	3.57	6.5	5.6	3.0	4.9
8-Br-guanosine	5.69	4.98	4.15	3.87	3.57	6.1	5.2	3.1	4.9
8-(α-Hydroxyisopropyl) guanosine	6.58	4.95	4.19	3.87	3.60	5.9	5.8	3.1	4.3

[a] Centre of bands H(5′) and H(5″).
[b] Mean value for *J*(4′,5′) and *J*(4′,5″).
[c] Overlapping of signals H(4′), H(5′), H(5″).

Table II. Calculated conformational parameters for the sugar ring, exocyclic group, and the base for guanosine and 5′-GMP and their 8-substituted analogues in aqueous medium and DMSO.

Nucleoside or Nucleotide	Populations [%][a]		
	syn	C(2′)*endo*	*gauche-gauche*
5′-GMP	30	58	63
8-NH_2-5′-GMP	30	74	80
8-CH_3-5′-GMP	80	64	41
8-Cl-5′-GMP	90	61	22
8-Br-5′-GMP	> 90	66	32
8-(α-Hydroxyisopropyl)-5′-GMP	100	58	[b]
Guanosine	40	64	63
8-NH_2-guanosine	40	76	75
8-CH_3-guanosine	70	69	54
8-Cl-guanosine	90	68	29
8-Br-guanosine	> 90	67	29
8-(α-Hydroxyisopropyl)-guanosine	100	68	45

[a] Estimated errors of sugar ring and exocyclic group conformations are 5%, and for conformations about the glycosidic bond 10%.
[b] Not calculated, because overlapping of appropriate signals of H(4′), H(5′) and H(5″) did not permit of determination of coupling constants.

midine ring and the lone pair on N(3), and are therefore dependent solely on the degree of preference for the conformation *syn*. One apparent exception to this is 8-aminoguanosine and 8-amino-5′-GMP, for which the changes in chemical shifts of H(2′) and H(1′) are relatively small compared to the other analogues.

The procedure for determination of the populations *syn* and *anti* in purine nucleosides and nucleotides, from an analysis of the chemical shifts of H(2′), has been described in detail elsewhere [7, 22]. For nucleosides this is based on a comparison of these values with the corresponding ones in model analogues constrained to the conformation *anti* by means of an intramolecular bond C(5′)−O−C(8), or to the conmformation *syn* by a sufficiently bulky C(8) substituent, such as 8-(α-hydroxyisopropyl) or 8-*tert*-butyl. In the case of the corresponding nucleotides, the *syn* and *anti* populations are derived from those evaluated for the parent nucleosides, and analysis of the chemical shifts of H(2′) for the nucleotides and their model *syn* analogues.

Application of the foregoing procedure to 5′-GMP and guanosine pointed to a preference for the form *anti* of 100% and 80% in aqueous medium and in DMSO-d_6, respectively. Further refinement of this approach, based on simultaneous measurements of ^{13}C chemical shifts, including C(2′), point to preferences for the forms *anti* of 70% for 5′-GMP and 60% for guanosine, to an accuracy of 10% (in preparation). A major difficulty in assignment of accurate values is due to the fact that, in the conformation *anti*, the 8-substituent may be close to H(2′) and thus additionally modify its chemical

shift. On the other hand, when there is a marked preference for the form *syn*, this effect is small (see Table II).

The compounds examined in this study may be assigned to three groups. The first includes guanosine, 5′-GMP and their 8-amino derivatives, which exhibit a preference for the conformation *anti*. Because of the effect of the 8-amino substituent on the chemical shift of H(2′) with the conformation *anti*, it is not feasible to evaluate the populations as accurately as for the the parent nucleoside and nucleotide. The differences between the chemical shifts of H(2′) in the 8-amino analogues, and for the parent nucleoside and nucleotide, 0.19 ppm and 0.06 ppm, are due to the 8-amino substituent. In the 8-amino analogues the conformation *anti* is also partially stabilized *via* an intramolecular hydrogen bond between the amino group and the exocyclic 5′-CH_2OH or 5′-$CH_2OPO_3^{-2}$ [23], which partially counteracts the unfavourable steric interaction between the amino substituent and the sugar ring. It may, nonetheless, be concluded that 8-aminoguanosine and its nucleotide exhibit a preference for the conformation *anti* of about 60%.

The second group includes the 8-methyl, 8-chloro and 8-bromo derivatives, for which preference for the form *syn* is quite marked, from 70% to more than 90%, respectively. The almost 30% population of the form *anti* for the 8-methyl analogues derives from the fact that, although the van der Waals' radius of the methyl group is similar to that of bromine, its non-spherical shape permits of closer interaction with the sugar ring, supported also by the results of theoretical calculations [22]. Even in the case of the 8-bromo analogues, the presence of a small proportion of the form *anti* ($< 10\%$) cannot be excluded, the more so in that, in the solid state complex of 8-bromo-ADP-ribose with a dehyrogenase, the adenine moiety is in the *anti* conformation [24]. Bearing in mind the van der Waals' radius of Cl is 1.75 Å, compared to 1.94 Å for Br [25], it is somewhat surprising that the 8-chloro analogues exhibit *syn* populations so close to those of the 8-bromo derivatives (Table II). This suggests that factors other than steric may also play some role.

The third group includes 8-(α-hydroxyisopropyl)-5′-GMP and its nucleoside, as well as 8-*tert*-butyl-5′-GMP (see ref. 6), where the large bulk of the 8-substituent excludes possible existence of the *anti* conformer.

Conformation of sugar rings. These were determined essentially according to Altona and Sundaralingam [26], and Guschlbauer [27]. It will be seen from Table 2 that all the nucleosides exhibit a preference for the C(2′)*endo* conformation (58–68%) which, in the case of the 8-amino derivatives, increases to 75%, because of intramolecular hydrogen bonding (see above).

Conformation of exocyclic groups. As normally encountered with other nucleosides, the exocyclic 5′-CH_2OH exists as a dynamic equilibrium of three classical rotamers, *gauche-gauche*, *gauche-trans* and *trans-gauche* [28]. Since, in most instances, it was possible to determine only the sum of $J(4',5')$ and $J(4',5'')$, only the *gauche-gauche* populations were evaluated (Table II). These varied from 63% for 5′-GMP and guanosine to 75–80% for the 8-amino derivatives, clearly because of intramolecular hydrogen bonding in the latter. It should, however, be noted that for the derivatives which are exclusively *syn*, the *gauche-gauche* population decreased to 30%.

Susceptibility to 5′-nucleotidase

Since 5′-GMP possesses a dissociable proton at the ring N(1) of the aglycone, with a pK of about 9.3, enzymatic hydrolysis was conducted at pH 7.5, where it is present, together with its analogues, predominantly in the neutral form. Following the observation that the 8-substituted analogues were very slowly hydrolyzed, rates of hydrolysis were compared quantitatively at a concentration of 2×10^{-3} M in the presence of 1.4 U/ml of 5′-NPase, with uncubation for 45 min at 37 °C, and removal of aliquots at 3-min intervals for assay of liberated P_i. From Table III which gives the initial rates of hydrolysis for each compound, it will be seen that an 8-subtituent has a decisive effect in reducing the rate of dephosphorylation. Under these conditions, an Eadie-Hofstee plot [29] yielded, for 5′-GMP, a $K_m = 0.8 \times 10^{-4}$ M, and a $V_{max} = 3.6 \times 10^{-5}$ M/min.

Several trials demonstrated that ribose-5-phosphate itself was not a substrate, in agreement with most, but not all (see below) previously reported observations. AcycloGMP, which has been shown to undergo hydrolysis very slowly in the presence of an excess of the enzyme [30], was resistant under the present conditions.

Each of the analogues, and two of the nucleosides, were then tested for potential inhibition of

Table III. Rates of dephosphorylation of 8-substituted analogues of 5′-GMP, relative to that for 5′-GMP, and % inhibition of 5′-GMP dephosphorylation in the presence of various concentrations of 8-substituted analogues.

Analogue	Rate of dephosphorylation (%)	% Inhibition		
		Inhibitor concentration [M]		
		2×10^{-3}	1×10^{-2}	2×10^{-2}
5′-GMP	100	–	–	–
8-methyl-5′-GMP	0.4	31	75	90
8-bromo-5′-GMP	1.5	28	–	75
8-chloro-5′-GMP	1.7	23	–	85
8-amino-5′-GMP	6.0	22	35	55
8-(α-hydroxyiso-propyl)-5′-GMP	0	23	–	35
8-*tert*-butyl-5′-GMP	0[a]	–	–	–
Guanosine	–	5	–	–
8-chloroguanosine	–	7	–	–
Acyclo GMP	0[b]	34	–	–
Ribose-5-phosphate	0	32	–	–

[a] From ref. [6].
[b] At much higher enzyme concentrations, this compound is very slowly dephosphorylated [30].

dephosphorylation of 5′-GMP. At concentrations equimolar with that of substrate, the 8-substituted nucleotides exhibited only moderate, and comparable, inhibitory properties. Only at 10-fold higher concentrations did several of them give appreciable inhibition, particularly 8-methyl-5′-GMP (Table III). Especially interesting was the finding that acyclo-GMP and ribose-5-phosphate, which were not substrates under these conditions, apparently inhibited to the same extent as the nucleotide analogues, at concentrations equimolar with that of substrate (Table III).

Because of the tediousness of the step-by-step assay of liberated P_i for 5′-nucleotidase, we have begun the development of a procedure for continuous assay of enzyme activity, based on the change in fluorescence of formycin-5′-phosphate accompanying its dephosphorylation (J. Wierzchowski *et al.*, in preparation). With the aid of this system, which is particularly well suited for studies on inhibitors which are not themselves fluorescent, we have found that the K_i of ribose-5-phosphate for inhibition of dephosphorylation of formycin-5′-phosphate is 0.2 mM at pH 7, and increases to about 2 mM at pH 9.1.

The appreciable increase in K_i for ribose-5-phosphate at alkaline pH probably accounts for the claim that this compound is neither a substrate, nor an inhibitor, at the pH 9 frequently employed for assay of this enzyme. It is, consequently, of interest that, during preparation of this manuscript, a report appeared by Sorensen and Butler [31] to the effect that ribose-5-phosphate is hydrolyzed by intestinal mucosa 5′-nucleotidase at pH 6.5 with a V_{max} comparable to that for 5′-AMP, but with a much lower K_m. Furthermore, the V_{max} for 5′-AMP increased 2½-fold, while the K_m increased 3-fold, when the pH was raised from 6.5 to 8. Unfortunately, these parameters were not determined for ribose-5-phosphate at pH 8. Nonetheless, the foregoing observations, and our own (see above), appear to indicate that ionization of the ribose-5-phosphate secondary hydroxyl (pK ~ 6.2) increases its binding to the enzyme, and its inhibitory properties. However, this problem requires more detailed investigation, since little attention has hitherto been devoted to a comparison of the properties of 5′-nucleotidases from different sources, *e.g.* the snake venom enzyme has been reported to slowly hydrolyze nucleoside 5′-methylphosphonates [32, 33], which are claimed to be resistant to the intestinal mucosa enzyme [31].

With the foregoing in mind, it is of interest to revert to the results listed in Table III. Not only are the rates of hydrolysis of 8-methyl-, 8-chloro- and 8-bromo-5′-GMP comparable, but their weak inhibitory properties (at concentrations equimolar with substrate) are also similar and comparable to that for ribose-5-phosphate. It therefore appears reasonable to assume that, in these analogues, one of the sites of recognition by the enzyme is the ribose-5′-phosphate moiety; whereas for the parent 5′-GMP in the conformation *anti*, it is the base which makes the more important, but not exclusive, contribution to substrate recognition. A parallel situation prevails for the 8-amino analogue, which readily adopts the *anti* conformation (Table II), but is hydrolyzed at a much lower rate because of the 8-amino substituent. Finally, it should be noted that the nucleosides themselves, like guanosine and 8-chloroguanosine, are even weaker inhibitors (Table III), if at all.

Concluding remarks

A comparison of the susceptibilities to 5′-NMPase of the various 8-substituted 5′-GMP analogues

(Table III) with their conformational parameters (Table II) indicates that enzymatic dephosphorylation proceeds, albeit slowly, only for those compounds capable of adopting, to some extent, the *anti* conformation about the glycosidic bond, the rates of hydrolysis varying from one to two orders of magnitude below that for the parent 5′-GMP. A similar situation prevails for 8-substituted 5′-AMP analogues, *e.g.* 8-bromo-5′-AMP which, like 8-bromo-5′-GMP, exhibits a very low population of the *anti* conformation [10], is a very weak substrate, whereas 8-(α-hydroxyisopropyl)-5′-AMP, which is incapable of adopting the *anti* conformation, is fully resistant. It has also been noted that 8-*tert*-butyl-5′-GMP, also exclusively in the *syn* conformation, is not a substrate [6]. It is, therefore, rather striking that 2-methylformycin-5′-phosphate, a structural analogue of 8-methyladenosine-5′-phosphate, is, like the latter, hydrolyzed by the same enzyme at only about 2% of the rate for the parent formycin-5′-phosphate (J. Wierzchowski *et al.*, in preparation).

The poor substrate properties of the 8-substituted analogues (including the 8-amino, which is preferentially *anti*, see Table II) must therefore be due to steric and other effects of the 8-substituents. The differences in rates of hydrolysis of these weak substrates are also related in part to the nature of the substituent, rather than the population of the *anti* conformer of the free nucleotide, since 8-bromo-5′-GMP (> 90% *syn*) is hydrolyzed more readily than 8-methyl-5′-GMP (80% *syn*).

One conceivable interpretation of the differences in rates of hydrolysis of the 8-methyl, 8-bromo, 8-chloro and 8-amino analogues is the hydrophobicity of the 8-substituent. Hydrolysis of the O(5′)-P linkage appears to involve an "in line" mechanism, with participation of a water molecule [5]. With the 8-substituent located at the active centre of the enzyme, and in the conformation *anti*, the substituent is in the vicinity of the phosphate group and, the greater its hydrophobicity, the more effectively it will prevent access of a water molecule from the medium. Hence the slowest rate of hydrolysis of the 8-methyl analogue, the methyl group being the most hydrophobic of these substituents. Even with the most hydrophilic substituent, NH_2, the rate of hydrolysis is very low relative to the parent 5′-GMP because of steric effects.

On the other hand, it appears that forced constrainment, by an 8-substituent as bulky as α-hydroxyisopropyl, to an exclusively *syn* conformation leads to extreme steric hindrance by the six-membered pyrimidine ring of the purine base, which is then located in the vicinity of the phosphate group, thus effectively hindering dephosphorylation.

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