Tautomerism of Isoguanosine and Solvent-Induced Keto-Enol Equilibrium

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Isoguanine Analogues, Isoguanosine, Tautomerism, Tautomeric Equilibrium, Solvent Polarity

Ultraviolet and infrared absorption spectroscopy, in aqueous and non-aqueous media, have been employed to study the tautomerism of 9-substituted isoguanines, including the nucleoside isoguanosine. With the aid of a series of model compounds, it was shown that 9-substituted isoguanines, and isoguanosine, in aqueous medium are predominantly in the form N(1)H,2-keto-6-amino.

In dioxane solution the tautomeric equilibrium is shifted in the direction of the enol form. The shift towards this form is accentuated for those analogues in which the exocyclic amino group is methylated.

With the aid of N^6,N^6,9-trimethylisoguanine, and its 9-octyl analogue, the tautomeric constant was studied as a function of concentration, temperature, and solvent polarity, and the results applied to evaluate the tautomeric equilibria of 9-methylisoguanine and isoguanosine as a function of these variables. In general the enol form is favoured by a decrease in solvent polarity, by a decrease in concentration in dioxane, or an increase in temperature in chloroform solution.

Syntheses are described for several N^6-amino and methylamino derivatives of 2-methoxy-9-methylpurine, and 3-methyl-5-oxo-7,8-dihydroimidazo(2,1-i)purine, which served as an analogue of the unavailable 1,9-dimethylisoguanine.

Considerable attention has been devoted to studies on the tautomerism of purine bases, in particular on the equilibrium between the N_7H and N_9H forms for hypoxanthine [1], guanine [2], adenine in *n*-butanol [3] and in aqueous medium [4], purine [5], and isoguanine in aqueous medium [6]. The results in these instances, involving tautomerism between ring nitrogens, are reasonably well established.

More difficult has been the problem of amino-imino and enol-keto tautomerism, and most of the evidence to date is consistent with the existence of purines largely in the amino and/or keto forms [7]. During the course of a study on the structure and conformation of poly isoguanylate [8] (poly isoG), it proved necessary to examine the possible tautomeric forms of isoguanosine in order to propose an acceptable base-pairing scheme which would satisfactorily account for the properties of this polymer, thus leading to the present investigation.

Although both isoguanine and isoguanosine have been isolated from natural sources [9, 10], they have not been found in natural nucleic acids. They do possess, in common with guanosine and its nucleotides, the interesting property of forming solution gels [11], which exhibit secondary structure *via* base pair hydrogen bonding similar to that found in helical polynucleotides.

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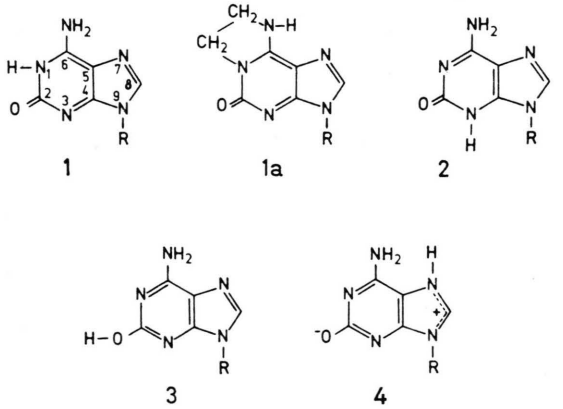
Abbreviations: N^6,N^6,9-m_3-isoG, N^6,N^6,9-trimethylisoguanine, N^6,N^6-m_2-9-octyl-iso-G, N^6,N^6-dimethyl-9-octylisoguanine; Hε-9-*m*-isoG, 3-methyl-5-oxo-7,8-dihydroimidazo(2,1-i)purine.

Materials and Methods

Isoguanosine was prepared by deamination of 2,6-diaminopurine riboside according to Davoll [12]. We are also indebted to Dr. G. B. Brown for an authentic sample of this compound. Some of the N-methyl derivatives of isoguanine were synthesized as



Scheme 1.

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elsewhere described [13]. It did not prove feasible to prepare 1,9-dimethylisoguanine *via* methylation and amination of 1,9-dimethyl-6-thioxanthine [14], since the latter underwent methylation only on N_7. This was consequently substituted for by another model compound, 3-methyl-5-oxo-7,8-dihydroimidazo(2,1-i)purine (Hε-9-*m*-isoG) (**1 a**, Scheme 1). Analogous derivatives have been previously employed for studies on tautomerism of cytosine [15]. N^6,N^6-dimethyl-9-octylisoguanine (N^6,N^6-m_2-9-octyl-isoG) was prepared from 9-octylxanthine *via* thiation and dimethylamination as described for other similar analogues [13].

3-methyl-5-oxo-7,8-dihydroimidazo(2,1-i)purine (**1 a**, Scheme 1)

This was prepared *via* N^6-hydroxyethyl-9-methylisoguanine as follows:

a. To a suspension of 500 mg of 9-methyl-6-methylthio-2-oxopurine [15] suspended in 60 ml *n*-butanol was added 1.5 ml anhydrous ethanolamine, and the mixture brought to reflux for 1 h. The crystals which separated out on cooling were collected by filtration to give 350 mg (73%) of N^6-hydroxyethyl-9-methylisoguanine, m.p. 265 – 267 °C.
Elementary analysis ($C_8H_{11}N_5O_2$):

Found: C 46.08% H 5.37% N 33.20%;
Calcd: C 45.93% H 5.26% N 33.49%.

b. A solution of 300 mg of N^6-hydroxyethyl-9-methylisoguanine in 15 ml thionyl chloride was heated under reflux for 30 min. The solution was then brought to dryness under reduced pressure and the residue dried under vacuum over KOH. The dried residue was taken up in 20 ml pyridine and heated under reflux for 30 min. The solution was brought to dryness, and pyridine removed by several evaporations of the residue from water. The resulting crude product was recrystallized from methanol to yield 225 mg (69%) of the HCl salt of 3-methyl-5-oxo-7,8-dihydroimidazo(2,1-i)purine (**1 a**) m.p. ~300 °C (decomp.).
Elementary analysis ($C_8H_9N_5O \cdot HCl$):

Found: C 42.59% H 4.05% N 30.89%;
Calcd: C 42.48% H 3.98% N 30.97%.

N^6-amino and methylamino derivatives of 2-methoxy-9-methylpurine

Each of these was prepared by a two-step procedure involving (a) methylation of the appropriate N^6-amino or methylamino 2-chloropurine, followed by (b) methoxylation of each of these, as follows:

a. To 1 mmol of 6-amino (or 6-methylamino) 2-chloropurine [17] dissolved in 15 ml dimethylformamide was added 1.1 mmol K_2CO_3. To this solution was added, with constant stirring and over a period of 1 hour, 1.5 mmol of methyl iodide, and stirring continued for an additional 30 min. The solution was brought to dryness under reduced pressure, and the crude product purified by chromatography on Merck PF_{254} silica gel with a solvent consisting of chloroform – methanol (95:5, v/v). The major band was eluted with 1:1 chloroform – methanol and, following removal of the eluent under reduced pressure, the residue crystallized from water. The products obtained from the three syntheses were as follows:

Derivative of 2-chloro-9-methylpurine	Yield [%]	m.p. [°C]	Analysis for N [%] Theor.	Measured
N^6-amino-	82	300	38.15	38.40
N^6-methylamino-	70	240–242	35.45	35.11
N^6-dimethylamino-	60	145–147	33.10	32.86

b. Each of the amino derivatives from the preceding section (0.5 mmol) together with 10 mmol CH_3ONa in 15 ml methanol was heated in a steel bomb at 140 °C for 5 hours. To the reaction mixture was added 100 ml water, and the solution passed through a 10 × 2 cm column of Dowex 50W × 8 (NH_4^+). The effluent was brought to dryness and the residue purified by chromatography on Merck PF_{254} silica gel, using as solvent chloroform – methanol (9:1, v/v). The major band was eluted with methanol, the methanol removed under reduced pressure, and the residue crystallized from aqueous methanol. The three products synthesized were as follows:

Derivative of 2-methoxy-9-methylpurine	Yield [%]	m.p. [°C]	Analysis for N [%] Theor.	Measured
N^6-amino-	62	271–273	39.04	39.22
N^6-methylamino-	48	189–190	36.26	35.93
N^6-dimethylamino-	40	135	33.82	33.91

Ultraviolet absorption spectra were run on a Zeiss (Jena, GDR) VSU-2P instrument and on a UNICAM Model 8000 recording spectrophotometer, using 10-mm pathlength cuvettes unless otherwise indicated. Spectral measurements at elevated concentrations were performed with variable path-length cuvettes fitted with CaF_2 windows.

Non-aqueous solvents included: reagent grade dioxane, distilled over sodium with constant passage

of a stream of dried nitrogen; reagent grade ethyl acetate; spectroscopic grade acetonitrile (Schuchardt, München, GFR); spectroscopic grade methanol (Chemopol, Praha, Czechoslovakia); reagent grade 99.8% ethanol; reagent grade chloroform, redistilled and stabilized with pentene[18]; DMSO (Eastman-Kodak), dried over CaH_2; formamide (AR, Austranal-Praparate, Loba Chemie, Wien).

Infrared absorption spectra were recorded on a Zeiss (Jena, GDR) double-beam model UR-10 spectrophotometer, using variable pathlength cuvettes fitted with CaF_2 windows for solutions in D_2O and dioxane, and cuvettes with NaCl windows for solutions in dioxane (600 μm pathlength). Solutions in $CDCl_3$ were measured in 5-mm pathlength quartz cuvettes. Solvents employed included: D_2O (>99.7 mol% D) from Koch-Light (England); reagent grade dioxane, as above, additionally dried with BDH (England) type 4A molecular sieves; $CDCl_3$ dried with Merck (Darmstadt, GFR) silicagel.

pH measurements made use of a Radiometer type PHM22 instrument with a G220B glass electrode. Buffers employed were acetate (pH 3 – 5), phosphate (pH 5.5 – 8) and Britton-Robinson (pH 8 – 11), all at concentrations of 0.01 M. 1 N, 0.1 N and 0.01 N HCl solutions were taken as pH 0, 1 and 2, respectively; while 0.01 N, 0.1 N and 1 N NaOH were taken as pH 12, 13 and 14. Although pH readings were usually obtained to an accuracy of 0.01 pH units, spectrophotometrically titrated pK values are accurate to about ±0.05 units.

Results and Discussion

Spectrophotometric titration, as elsewhere described[13], was employed to determine the pK values for protonation and/or dissociation of the isoguanine derivatives. The resulting values, shown in Table I,

Table I. Spectrophotometrically determined pK values at 20 °C for protonation and dissociation of some isoguanine analogues and isoguanosine.

Compound	pK_1	pK_2
isoguanosine	3.4	9.8
9-methylisoguanine	3.85	9.9
3,9-dimethylisoguanine	4.4	—
H ε-9-*m*-isoG	3.8	11.4
2-methoxy-6-amino-9-methylpurine	3.5	—
2-methoxy-N^6,N^6,9-trimethylpurine	3.4	—
N^6,N^6,9-m_3-isoG	3.9	9.9

were taken account of in spectral measurements to avoid the presence of an admixture of neutral and ionic forms.

The low pK_1 values, clearly corresponding to protonation, of the compounds listed in Table I, and for which amino-imino tautomerism is possible, suggests that these exist in aqueous medium largely in the amino form. Consequently, in the case of 9-methylisoguanine (and isoguanosine), four possible tautomeric forms are possible, **1**, **2**, **3** and **4** (Scheme 1). However, the zwitterionic form **4** may be excluded from consideration, since it would be expected to exhibit a pK_1 of about 7, in accordance with the reported properties of the zwitterion of N_7H^+ of 9-methylguanine[2]. We are therefore left with the three possible forms N_1H (**1**), N_3H (**2**) and O_2H (**3**) for 9-substituted isoguanines (Scheme 1).

The absorption spectra of the neutral forms of isoguanosine, 9-methylisoguanine and several other model compounds, in aqueous medium, are exhibited in Fig. 1A. As might have been anticipated,

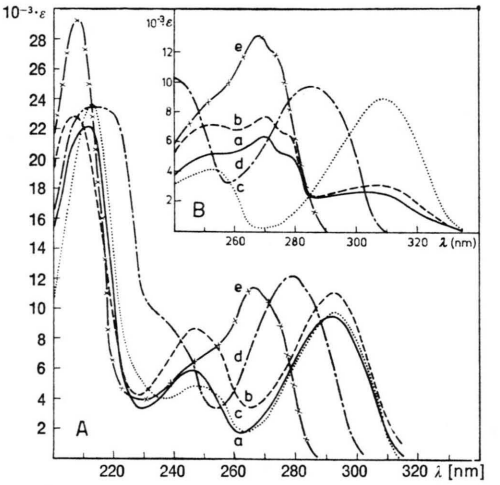


Fig. 1. Ultraviolet absorption spectra, (A) in neutral aqueous medium, (B) in dioxane containing 0.5% H_2O, of 0.5×10^{-4} M solutions of: a. 9-methylisoguanine; b. isoguanosine; c. Hε-9-methylisoguanine; d. 3,9-dimethylisoguanine; e. 2-methoxy-9-methyladenine.

isoguanosine and 9-methylisoguanine exhibit three common bands with maxima at about 290, 250 and 210 nm, differing only somewhat in intensities; furthermore, the spectrum of Hε-9-*m*-isoG which may be considered an analogue of 1,9-dimethylisoguanine, bears a very close resemblance to that of 9-methylisoguanine. For both 3,9-dimethylisoguanine and 2-methoxy-9-methyladenine the principal long wavelength bands are shifted appreciably to the violet (relative to 9-methylisoguanine), while the bands at about 250 nm are not clearly defined. The spectral differences between the first three analogues on the one hand, and the remaining two on the other, are sufficiently pronounced as to exclude for

9-methylisoguanine the tautomeric form N_3H (**2**) or the enol form (**3**). The marked similarity of the spectra of 9-methylisoguanine and Hε-9-*m*-isoG (**1a**) points to the existence of the former in the form N_1H. This would also be the tautomeric form of isoguanosine, if we assume that the differences in intensities of the isoguanosine bands from those for 9-methylisoguanine are due to replacement of the 9-methyl substituent by ribose.

UV absorption spectra in dioxane. Spectral changes resulting from a change in the solvent medium may be associated with a shift in tautomeric equilibrium [19] or some specific interaction with solvent molecules [20]. In the present instance effects due to possible autoassociation may be discounted because of the low solute concentrations employed, about 10^{-4} M.

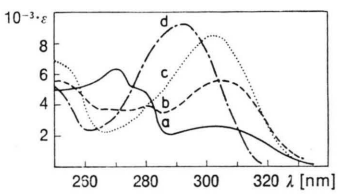


Fig. 2. Ultraviolet absorption spectrum of 9-methylisoguanine, 0.5×10^{-4} M, in dioxane with a water content of a. 0.5%; b. 2.7%; c. 7.2%; and d. in aqueous neutral medium.

Fig. 1B exhibits the absorption spectra of the same series of compounds as in Fig. 1A, but in dioxane with addition of 0.5% H_2O, which is necessary to provide adequate solubility. It will be seen that both isoguanosine and 9-methylisoguanine undergo very marked changes on transfer from water to dioxane. They exhibit two new bands, one about 20 nm to the red of the 290 nm band in water; the other, showing some structure, about 20 nm to the violet of 290 nm. Of the other three compounds, the spectrum of 2-methoxy-9-methyladenine is practically unaffected; whereas the long wavelength bands of 3,9-dimethylisoguanine and Hε-9-*m*-isoG are appreciably red shifted and their extinction slightly decreased.

Pronounced red shifs of the longwavelength absorption bands, resulting from transfer from water to a non-polar medium, such as dioxane, have been observed and their origin discussed in the case of monocyclic hydroxyazines [21] and, more recently, cytidine [20]. These are not necessarily a reflection of shifts in tautomeric equilibrium. As pointed out [20], it is not the change in solvent polarity which is mainly responsible for this effect, but whether the change in solvent is to one which is protic or aprotic, so that the observed band shift is most likely due to hydrogen bonding between solute and solvent. The same authors conclude that it is the cytidine ring N_3 which is involved in such hydrogen bonding, since this is the position which is protonated on cation formation. It is, however, unlikely that such is the case, since protonation of cytidine on N_3 results in a red shift of the long wavelength absorption band [22]. It appears more likely that hydrogen bonding occurs to the carbonyl, as proposed for cytosine [23]. This appears to us to be the reason for the shift to the violet of the long wavelength absorption bands of 3,9-dimethylisoguanine and Hε-9-*m*-isoG on transfer from dioxane to water (Figs 1A and 1B), accompanied by hydrogen bonding of water molecules to the C_2 carbonyl, with maintenance of the amino forms for both compounds. The similarity of the spectra of 2-methoxy-9-methyladenine in water and dioxane (absence of a carbonyl group and of any change in band location) is in accord with the foregoing assumed role of the carbonyl group in the observed band shift, and also testifies to maintenance of the amino form of the compound. The appropriate tautomeric forms of isoguanosine and 9-methylisoguanine would be expected to behave like the above model compounds.

Amino-imino tautomerism may be excluded as the source of the structured bands at 270 nm, in dioxane, for isoguanosine and 9-methylisoguanine, since analogues effects are observed for N^6,N^6,9-m_3-isoG (Fig. 3). If we compare the spectra of isoguanosine and 9-methylisoguanine with those for the model compounds corresponding to the three pos-

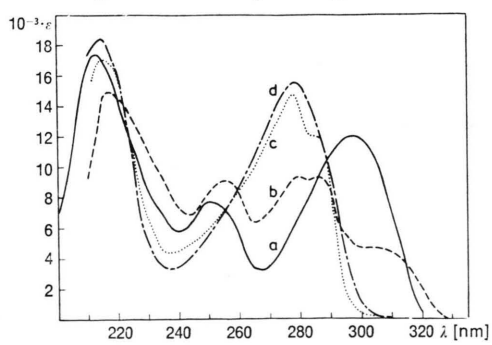


Fig. 3. Absorption spectra of N^6,N^6-9-m_3-isoG in a. neutral aqueous medium, b. in ethanol, c. in dioxane, and d. of 2-methoxy-9-methyladenine in neutral aqueous medium. *Note*: the spectrum in dioxane was run on a 10-fold higher concentration in a 1-mm pathlength cuvette to permit measurements to 210 nm.

sible tautomeric forms (Fig. 1B), it will be seen that these may be regarded as composites of the two tautomeric forms N_1H (**1**), which is responsible for the long wavelength band at about 310 nm, and the enol form **3** which gives rise to the short wavelenght structured band at about 270 cm. The ratio of the intensities of these bands is strongly dependent on the water content of the dioxane solution. For 0.5% H_2O in dioxane (Fig. 1B) the ratio of enol to keto forms for isoguanosine and 9-methylisoguanine may be evaluated as about 5:2, assuming the N_1H form to have the same extinction coefficient at the absorption maximum as Hε-9-*m*-isoG.

An increase in the water content of the dioxane solution leads to a decrease in extinction of the band corresponding to the enol form, with a simultaneous increase in intensity of the band corresponding to the form N_1H and a marked shift to the violet (Fig. 2). For a water content of about 7% the band corresponding to the enol form has virtually disappeared, although that corresponding to the form N_1H is still displaced from its position in aqueous medium. An increase in the water content led to a further shift of this band towards the violet. Because of the simultaneous change in location of the long wavelength absorption band of the form N_1H, and the tautomeric equilibrium, no isosbestic point is observed. Since the proportion of the keto form increases quite rapidly with increasing water content, it may be assumed that this form is more highly stabilized because of its higher polarity. This is supported by both theoretical calculations, and experimental measurements of dipole moments (in preparation) and is in accordance with the properties of other lactam and lactim forms in tautomeric equilibrium [24].

Keto-enol tautomerism of N^6,N^6,9-m_3-isoG. In view of the relatively poor solubilities of isoguanosine and 9-methylisoguanine in dioxane, where enolization appeared to be quite marked, recourse was had to N^6,N^6,9-m_3-isoG. This analogue possesses the additional advantage that it is in the fixed amino form. Its pK for protonation is almost identical with that for 9-methylisoguanine (Table I), excluding its existence in the form of a zwitterion [2]. Its structure may therefore be considered in relation to the three tautomeric forms already considered for isoguanosine, above. Although we did not disponse of the two model compounds with the forms N_1H and N_3H, the fixed enol form 2-methoxy-N^6,N^6,9-trimethylpurine was available.

Fig. 3 exhibits the spectra, in neutral aqueous medium, of N^6,N^6,9-m_3-isoG and 2-methoxy-N^6,N^6,9-trimethylpurine. The former exhibits three characteristic bands at 210, 250 and 297 nm, the latter two bands at 214 nm and 278 nm. The differences are so pronounced as to practically exclude the presence of the enol form in N^6,N^6,9-m_3-isoG. We are therefore left with the two forms N_1H and N_3H. In the absence of the N_1-methyl and N_3-methyl derivatives of N^6,N^6,9-m_3-isoG, the spectrum of the latter was compared with the corresponding model compounds for 9-methylisoguanine. Since methylation of the amino group normally leads to a bathochromic shift of the spectrum, the resemblance of the spectrum of N^6,N^6,9-m_3-isoG to that of Hε-9-*m*-isoG (Figs 1A and 3) points to the existence of the former predominantly in the form N_1H.

Attention was then directed to the spectrum of N^6,N^6,9-m_3-isoG in solvent of various polarities (Fig. 3). In dioxane, and also in dioxane with 0.5% H_2O, there is a structured band at about 277 nm, and a second, short-wavelength, band at 216 nm. The location of these bands corresponds to those of 2-methoxy-N^6,N^6,9-trimethylpurine in neutral aqueous medium (Fig. 3) (or in other solvents), indicating that N^6,N^6,9-m_3-isoG exists largely in the enol form in dioxane. Comparison of the spectra of N^6,N^6,9-m_3-isoG and 9-methylisoguanine in dioxane (Figs 1B and 3) shows that their structured absorption bands at 277 nm and 270 nm are strikingly similar. Since 9-methylisoguanine in dioxane with 0.5% H_2O has already been shown to include the enol and N_1H forms, it appears that methylation of the amino group leads to a shift in the tautomeric equilibrium towards the enol form. An increase in the water content of the dioxane solution, or a change to a solvent more polar than dioxane (Fig. 3, the spectra in ethanol) leads to a decrease in the intensity of the structured 277 nm band of N^6,N^6,9-m_3-isoG, and the appearance of a new long wavelength band, as in the case of 9-methylisoguanine in dioxane.

Notwithstanding the lack of suitable fixed model compounds for the forms N_1H and N_3H of N^6,N^6,9-m_3-isoG, which makes it difficult to establish with certainty which form is responsible for the long wavelength absorption band, the similarity in behaviour to that for 9-methylisoguanine argues in favour of the tautomeric form N_1H.

Tautomeric constants and solvent polarity parameters. For a series of heterocyclic compounds, such as analogues of pyridone, it was shown [19] that the logarithm of the tautomeric constant for keto-enol equilibrium exhibited a linear dependence on the solvent parameter Z initially introduced by Kosower [25] from a study of the solvatochromic effect for the charge-transfer electronic transition of 1-alkylpyridinium iodides. The polarity parameter $E_{T_{30}}$ was subsequently introduced [26] on the basis of the solvatochromic effect for diphenyl-betaine, which is also a linear function of the parameter Z for a variety of solvents. The parameter $E_{T_{30}}$ was determined for a broader group of solvents, profiting from the superior solubility of diphenyl-betaine relative to 1-alkylpyridinium iodides.

With a view to determining the dependence of the tautomeric constant K_T for the keto-enol equilibrium in N^6,N^6,9-m_3-isoG on solvent polarity, use was made of a number of solvents with different parameters $E_{T_{30}}$ as established [26]. The solvents selected, their $E_{T_{30}}$ values, the location of the long wavelength absorption maximum λ_L, the corresponding extinction coefficient ε_L for the tautomer N_1H, and the limiting values for the logarithms of the keto-enol equilibrium constants determined from the UV spectra of N^6,N^6,9-m_3-isoG, are exhibited in Table II.

Table II. Calculated values of log K^+ and log K^- for the keto-enol equilibrium of N^6,N^6,9-m_3-isoG at a concentration of about 0.5×10^{-4} M in a number of solvents with different polarity parameters E_{T30} taken from Dimroth *et al.* [26]. The log K values were calculated as described in the text, using the measured value of the molar extinction coefficient, ε_L, at the peak, λ_L, of the long wavelength absorption band in each solvent at 20 °C.

N°	Solvent	E_{T30} [kcal/mol]	λ_L [nm]	ε_L	log K^+	log K^-
1	dioxane	36	315	100	−2.0	−2.14
2	ethyl acetate	38.1	314	700	−1.12	−1.28
3	chloroform	39.1	310	7100	0.39	0.01
4	DMSO	45	315	1500	−0.75	−0.92
5	acetonitrile	46	311	3800	−0.21	−0.43
6	ethanol	50.8	306	4700	−0.05	−0.30
7	methanol	55.5	305	6800	0.33	−0.02
8	formamide	56.6	304	8300	0.69	0.16
9	H_2O	63.1	287	12000	∞	0.78

Evaluation of the keto-enol equilibrium constants requires several additional assumptions, since precise values for the molar extinctions of the tautomeric forms are not directly available. It is most convenient to establish the ratio of tautomers from the value for the N_1H-keto form, which exhibits long wavelength absorption reasonably well resolved from the absorption region of the enol form (Fig. 3). From the data for 2-methoxy-N^6,N^6,9-trimethylpurine, the location of the long wavelength band of this fixed enol form should be relatively independent of the nature of the solvent. The content of the form N_1H-keto was estimated on the assumption that its molar extinction at its λ_{max} in various solvents is 12000 ± 2000. This appears reasonable in view of the fact that ε_{max} for the model compound Hε-9-*m*-isoG (Figs 1A and 1B) is not much different in water (9600) from that in dioxane (8800). It follows that the ratio of the extinction coefficient ε_L for the absorption maximum at λ_L to the value $\varepsilon = 12000\pm2000$ provides upper and lower limits for the content of the keto tautomer. The upper and lower limits for the value of the equilibrium constant would then be

$$K_T = \frac{(\text{keto})}{(\text{enol})} \quad \text{or} \quad K_T{}^+ = \frac{\varepsilon_L}{10000-\varepsilon_L},$$

$$K_T{}^- = \frac{\varepsilon_L}{14000-\varepsilon_L}.$$

The results are listed in Table II and exhibited graphically in Fig. 4. The vertical lines embrace the limiting values of the logarithms of the equilibrium

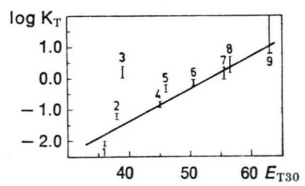


Fig. 4. Dependence of the tautomeric equilibrium constant, K_T, on the solvent polarity parameter E_{T30} for N^6,N^6,9-m_3-isoG, using the data from Table II.

constants listed in Table II. It will be noted from Fig. 4 that only in the case of chloroform do the values fall outside the linear relationship. This departure from linearity in the case of chloroform has also been observed with other tautomeric systems [27]. From the relatively good linear relationship prevailing for the remaining points, it may be concluded that $\log(K_T)$ is linearly dependent on $E_{T_{30}}$ and that the equilibrium shifts in the direction of the keto form with increase in solvent polarity. From this we may now estimate the content of the tautomer in aqueous medium, for which the equilibrium constant, in the range $(0.78, +\infty)$ is, in fact, indeterminate (Table II). Since no anomaly has pre-

viously been reported for water [19, 25], it appears reasonable to take $\log K_T = 1$, determined by the intersection of the vertical line No. 9 with the line representing the dependence of $\log K_T$ on $E_{T_{30}}$ (Fig. 4). This leads to a content of the enol form in water of about 10%, a proportion too small to place in direct evidence from an analysis of the UV absorption spectrum.

Temperature dependence of K_T

The tautomeric equilibrium might be expected to be dependent on the temperature. For N^6,N^6,9-m_3-isoG in ethanol, no shift in equilibrium was observed over the temperature range 15 – 70 °C, based on observation of the UV spectrum. In chloroform, however, a definite shift in equilibrium towards the enol form is evident with increasing temperature (Fig. 5). The resulting dependence of $\log K_T$ on

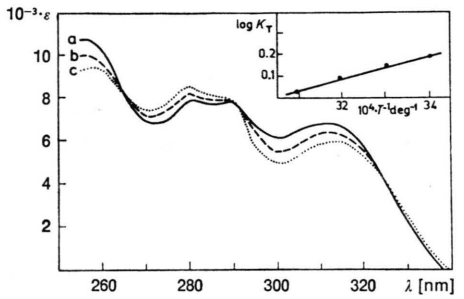


Fig. 5. Ultraviolet absorption spectrum of N^6,N^6,9-m_3-isoG, 10^{-4} M in chloroform at a. 30 °C; b. 40 °C; c. 50 °C. The insert shows the dependence of $\log K_T$ on the reciprocal of the temperature in °K.

$1/T$ is shown in the insert to Fig. 5. The values of K_T were determined as described above, taking $\varepsilon = 12 \times 10^3$, except that a correction was applied to each value at a given temperature for the decrease in concentration resulting from the temperature expansion of the solution. Since the points fall on a straight line (Fig. 5), the enthalpy for the tautomeric transition may be assumed constant over the temperature range employed, and is $\Delta H = 2.5 \pm 0.5$ kcal/mol, so that the transition from the enol to the keto form is, in this instance, exothermic.

In the case of pyridone-2 analogues, an extensive study of the temperature dependence of K_T in different solvents [28] demonstrated that the change in enthalpy was found to be markedly dependent on the nature of the solvent, and did not furnish any concrete information about the ΔH for the tautomeric transition of the isolated molecules in, *e. g.* the gas phase.

Concentration dependence of UV absorption spectra

In view of the possible concentration dependence of the tautomeric equilibrium, the UV absorption spectrum of the neutral form of N^6,N^6,9-m_3-isoG in aqueous medium was recorded at concentrations of 0.5×10^{-4} M and 4×10^{-2} M (Fig. 6A). Analogous

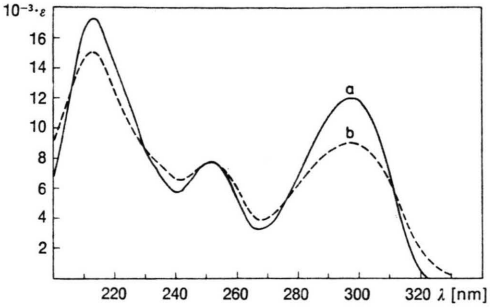


Fig. 6A

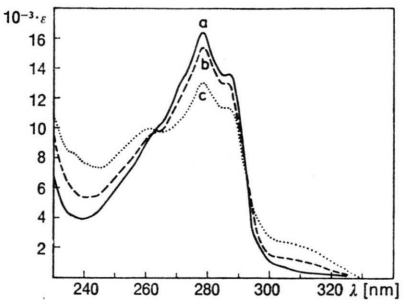


Fig. 6B

Fig. 6. Absorption spectra of N^6,N^6,9-m_3-isoG: (A) in neutral aqueous medium, a. 0.5×10^{-4} M (cuvette pathlength 10 mm) b. 4×10^{-2} M (cuvette pathlength 13 μm). (B) in dioxane, a. 0.5×10^{-4} M (pathlength 10 mm); b. 6×10^{-3} M (pathlength 40 μm); c. 4.2×10^{-2} M (pathlength 13 μm).

measurements in dioxane were carried out with N^6,N^6-m_2-9-octyl-isoG (because of its higher solubility in this solvent) at concentrations of 0.5×10^{-4} M, 6×10^{-3} M and 4.2×10^{-2} M (Fig. 6B).

From Fig. 6A it will be seen that an increase in concentration in aqueous medium led to a marked decrease in extinction (hypochromicity) of the principal bands at 210 and 300 nm, with no modification in the shapes of these bands. A similar hypochromic effect was noted for the principal long-wavelength absorption band of deoxyadenosine in aqueous medium [29] when the concentration was increased stepwise to 5×10^{-2} M; and this was shown by means of sedimentation equilibrium to be due to self-association under these conditions. The absence of any marked changes in location, or form, of the N^6,N^6,9-m_3-isoG bands with increase in concentra-

tion indicates that the tautomeric equilibrium is not affected.

In contrast to the foregoing, an increase in concentration in dioxane, particularly above 6×10^{-3} M, appreciably affects the major absorption bands (Fig. 6B). Two new bands make their appearance at 310 nm and 260 nm. The observed modifications in absorption spectrum are analogous to those resulting from an increase in solvent polarity for $N^6,N^6,9$-m_3-isoG (Fig. 3), interpreted above as a consequence of the shift in tautomeric equilibrium towards the keto form.

Infrared spectroscopy

With a view to independently checking the validity of the conclusions derived from the UV spectra, attention was next directed to the infrared absorption spectra of some of the analogues.

Figs 7A and 7B exhibit the infrared absorption spectra in neutral aqueous medium, and in anhydrous dioxane, of $N^6,N^6,9$-m_3-isoG and its 2-methoxy derivative, the latter representing the fixed enol form. The latter, as might be anticipated, exhibits no carbonyl absorption; there is an intense band at 1607 cm^{-1} in water, which is shifted to 1598 cm^{-1} in dioxane, and two weaker bands at 1460 cm^{-1} and 1507 cm^{-1} in water, which are also visible in dioxane (only the 1507 cm^{-1} band is noted in dioxane, which itself cuts off at about 1500 cm^{-1}).

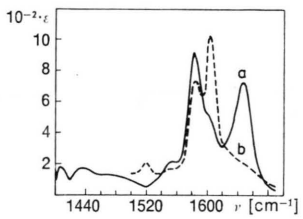


Fig. 7A

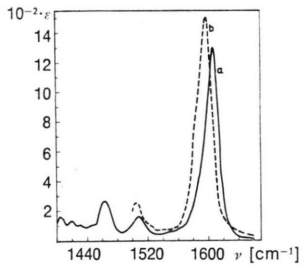


Fig. 7B

Fig. 7. Infrared absorption spectra of: (A) $N^6,N^6,9$-m_3-isoG, a. 7.6×10^{-2} M (pathlength 50 μm) in D_2O, pD 6.5; b. 5×10^{-3} M (pathlength 600 μm) in dioxane. (B) 2-methoxy-$N^6,N^6,9$-trimethylpurine, a. 3.4×10^{-2} M (pathlength 100 μm) in D_2O, pD 6.5; b. 10^{-2} M (pathlength 400 μm) in dioxane.

For $N^6,N^6,9$-m_3-isoG in aqueous medium, there is a strong band at 1645 cm^{-1} (Fig. 7A), which can be ascribed only to a carbonyl stretching frequency. Similar bands have been observed in this frequency range, and assigned to the carbonyl group, for pyrimidone-2 in aqueous medium[30], 1644 cm^{-1}; for 2-oxo-9-methylpurine in chloroform, 1637 cm^{-1}, and in the solid state[1], 1647 cm^{-1}; and for isoguanosine in aqueous medium[11], 1652 cm^{-1}. There is a second intense band at 1584 cm^{-1}, and a weaker band on the high frequency shoulder of the latter, at about 1605 cm^{-1}.

In sharp contrast to the fixed enol form (the 2-methoxy-derivative, Fig. 7B), $N^6,N^6,9$-m_3-isoG exhibits striking modifications in its absorption spectrum on transfer from water to dioxane (Fig. 7A). The intensity of the 1645 cm^{-1} band decreases dramatically, those at 1584 cm^{-1} and 1605 cm^{-1} reverse their relative intensities, and a new weak band appears at 1520 cm^{-1}.

The presence, in the spectrum of $N^6,N^6,9$-m_3-isoG in D_2O, of an intense band in the range of carbonyl frequencies, and the differences between this spectrum and that for the 2-methoxy derivative (Figs 7A, 7B), clearly point to the existence of this compound in aqueous medium largely in the keto form. The presence of a minor fraction in the enol form is suggested by the presence of a weak band at 1605 cm^{-1}, corresponding to the intense band at 1607 cm^{-1} in the 2-methoxy derivative. It is technically difficult to examine the concentration dependence of an IR spectrum in aqueous medium over a wide range of concentrations. However, since the concentration dependence of the UV absorption spectrum in aqueous medium points to the absence of a modification in tautomeric equilibrium at concentrations up to near those employed for recording infrared spectra (see Fig. 6A), the infrared measurements are consistent with the conclusion derived from the UV spectral data regarding the predominance in aqueous medium of the keto form.

For $N^6,N^6,9$-m_3-isoG at 5×10^{-3} M in dioxane (Figs 7A, 7B), the spectrum is similar to that for the 2-methoxy derivative, in agreement with the UV spectral data (Fig. 3), and with the existence of this compound under these conditions largely in the enol form. The presence of a small proportion in the keto form is suggested by the presence of the weak band

at about 1630 cm^{-1}. In this instance, however, account must be taken of the concentration dependence of the UV spectrum of N^6,N^6-m_2-9-octyl-isoG in dioxane, pointing to a shift in tautomeric equilibrium towards the keto form with an increase in concentration (see Fig. 6B).

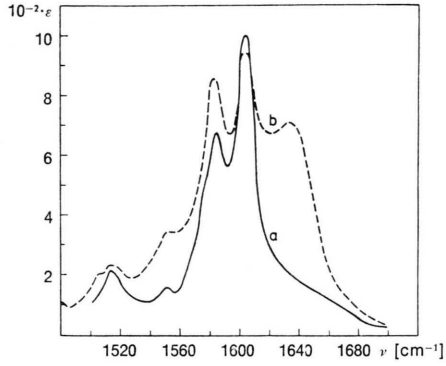
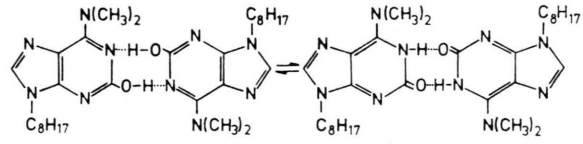


Fig. 8. Infrared absorption spectrum in dioxane solution of N^6,N^6-m_2-9-octyl-isoG at concentrations of a. 7×10^{-3} M (pathlength 600 μm); b. 7.5×10^{-2} M (pathlength 60 μm).

Fig. 8 exhibits the IR spectrum of the 9-octyl derivative in dioxane at concentrations of 7×10^{-3} M and 7.5×10^{-2} M. The spectrum at the lower concentration is very similar to that for $N^6,N^6,9$-m_3-isoG at 5×10^{-3} M (Fig. 7A), as was to be excepted. At the higher concentration there is a definite change in the spectrum; the weak broad band at about 1630 cm^{-1} increases in intensity, while that a 1604 cm^{-1} decreases in intensity. These modifications in the spectrum, in particular the appearance of a band in the range of carbonyl frequencies with an increase in concentration, are in accord with the conclusions derived from an examination of the UV absorption spectra, *viz.* a shift of the tautomeric equilibrium towards the keto form.

In the case of pyridone-2 in dioxane, Krackov *et al.*[31] demonstrated, with the aid of osmometric methods, that dimer formation, about 9.5% at a concentration of 0.013 M, increased to 56% at a concentration of 0.1 M. If we assume that N^6,N^6-m_2-9-octyl-isoG associates at elevated contentrations to form hydrogen bonded dimers, such dimers may be formed from either the enol or keto forms, as illustrated in Scheme 2. As shown above, the keto form of N^6,N^6-9-m_3-isoG is stabilized by an increase in solvent polarity. It therefore appears likely that the "keto" form of the dimer of N^6,N^6-m_2-9-octyl-isoG will be more stable than the "enol" form. The observed spectral modifications would then be the



Scheme 2.

consequence of an increase in "keto" dimer formation as the concentration is increased.

Infrared spectrum in chloroform

Because of its well-known properties as a proton acceptor, dioxane is not suitable as a solvent system for measurements of free N-H and O-H frequencies. Recourse was then had to chloroform. In this solvent both $N^6,N^6,9$-m_3-isoG and N^6,N^6-m_2-9-octyl-isoG exhibit low intensity bands at 3430 cm^{-1} and 3580 cm^{-1}. Deuteration of the latter compound (by vigorous shaking of a deuterochloroform solution with D_2O, followed by removal of D_2O with silicagel) led to disappearance of both these bands (Fig. 9). From

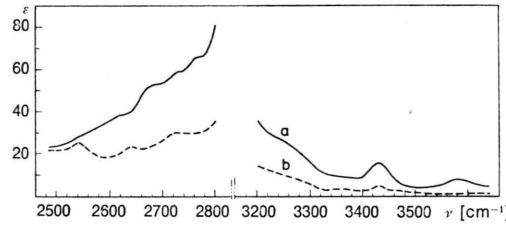


Fig. 9. Infrared absorption spectrum of N^6,N^6-m_2-9-octyl-isoG in $CDCl_3$: a. non-deuterated, 2×10^{-2} M (pathlength 5 mm); b. deuterated, 1.5×10^{-2} M (pathlength 5 mm).

this it may be concluded that the 3430 cm^{-1} band is due to an N-H stretch, and that at 3580 cm^{-1} to an O-H stretching frequency. The low intensities of these bands (the extinctions of which were found to increase somewhat with dilution, and with an increase in temperature), and the decrease in intensity of the broad band at frequencies above 2500 cm^{-1} following deuteration (rendering difficult identification of the corresponding N-D and O-D bands), point to pronounced self-association under these conditions. Similar self-association has been reported for 6-chloropyridone-2, which also exhibits keto-enol equilibrium in chloroform solution[32].

Concluding remarks

From the overall results it may be concluded that the neutral forms of 9-methylisoguanine and isoguanosine in aqueous medium are in the tautomeric

form N(1)H,2-keto-6-amino. This is similar to the tautomeric form proposed for 6-methylthio-2-keto-9-methylpurine on the basis of an analysis of the UV absorption spectra for a number of analogues [14].

This tautomeric form for neutral isoguanosine, in particular the location of the proton on the ring N_1, provided the basis for a proposed structure of the multi-stranded form of poly(isoG) consistent with the observed properties of the latter. This structure is believed to consist of two equivalent double helices linked by two supplementary hydrogen bonds to form a planar array of four hydrogen-bonded isoguanine residues with C_2 symmetry [8].

It is perhaps also worth noting that the findings of the present study, on the keto-enol equilibrium of 9-substituted isoguanines in solvents of low polarity, represent, to our knowledge, the first reported instance of the existence of oxopurines in the enol form in solution. It would clearly be of interest to extend these observations to other oxopurines, as well as to oxopyrimidines.

We are indebted to Anna Psoda for assistance with the infrared spectra. This investigation was partially supported by the Polish Academy of Sciences (Project 09.3.1 and 03.10.7.02.01) and The Wellcome Trust.

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