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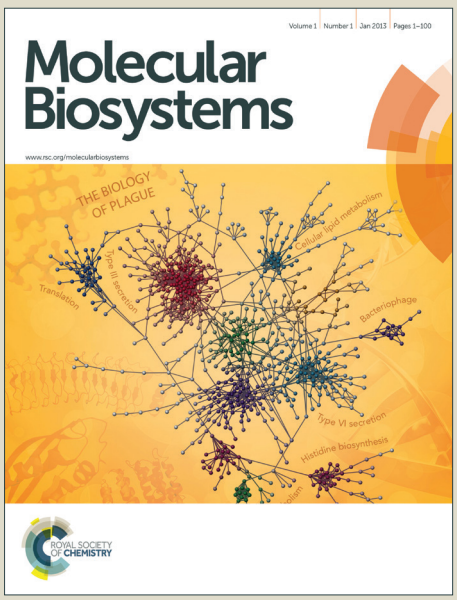

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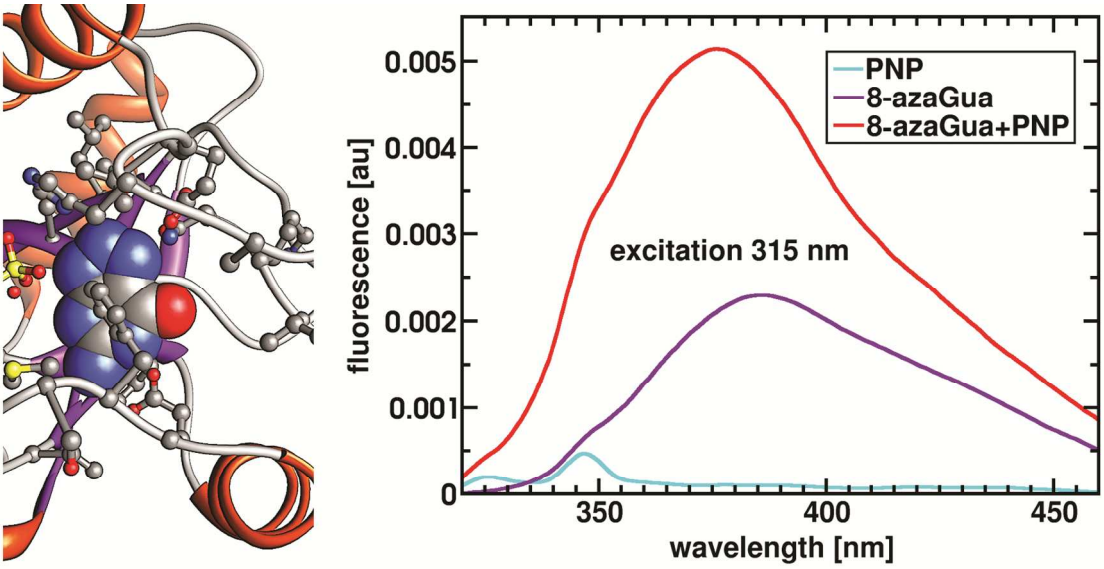

We review fluorescence emission properties of 8-azapurines and related compounds, and their potential as fluorescence probes in various biochemical systems.

8-Azapurines as isosteric purine fluorescent probes for nucleic acid and enzymatic research

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The 8-azapurines, and their 7-deaza and 9-deaza congeners, represent a unique class of isosteric (isomorphic) analogues of the natural purines, frequently capable of substituting for the latter in many biochemical processes. Particularly interesting is their propensity to exhibit pH-dependent room-temperature fluorescence in aqueous medium, and in non-polar media. We herein review the physico-chemical properties of this class of compounds, with particular emphasis on the fluorescence emission properties of their neutral and/or ionic species, which has led to their widespread use as fluorescent probes in enzymology, including enzymes involved in purine metabolism, agonists/antagonists of adenosine receptors, mechanisms of catalytic RNAs, RNA editing, etc. They are also exceptionally useful fluorescent probes for analytical and clinical applications in crude cell homogenates.

1 Introduction

One class of structural analogues of natural purines comprises the 8-azapurines, in which the C(8) of a natural purine is replaced by nitrogen, so that they are isosteric (isomorphic) with the parent purine. Our attention was initially directed to the 8-azapurines (1,2,3-triazolo[4,5-d]pyrimidines), the chemistry of which was earlier reviewed by Albert,[1] following the finding that 8-azaguanine (8-azaGua‡) is a substrate for the reverse synthetic pathway of purine nucleoside phosphorylase (PNP), and that the intrinsic fluorescence properties of this analogue and its nucleoside could be utilized both to monitor the reaction and its kinetics, as well as to study its mode of interaction with the enzyme.[2–6]

Subsequent studies revealed that other 8-azapurines, and their nucleosides and nucleotides, exhibit measurable emission in the neutral and/or ionic forms, of obvious utility in studies on nucleic acids and ligand/protein interactions.[7–17] The importance of fluorescent analogues in this field[18–28] has been highlighted by a special issue of Tetrahedron devoted to this subject, edited by Y. Tor[24] and more recent reviews by Sinkeldam et al.,[27] and Tanpure et al.[29] Somewhat surprisingly, these do not include the 8-azapurines and their nucleosides, the most closely related to natural purines, in terms of

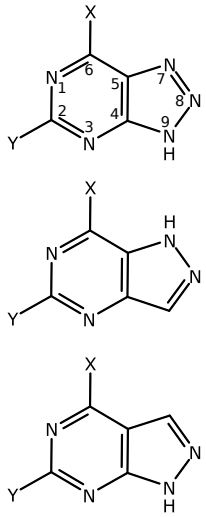

Fig. 1 Structures of 8-azapurines (top), 8-aza-9-deazapurines (pyrazolo[4,3-d]pyrimidines, middle), and 8-aza-7-deazapurines (pyrazolo[3,4-d]pyrimidines, bottom). All three exhibit prototropic tautomerism, not shown here, but discussed below. Throughout, the IUPAC numbering system is replaced, for the sake of uniformity, by purine numbering (X, Y = H, or O, or NH_2).

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‡Abbreviations: PNP, purine nucleoside phosphorylase; R1P, β-D-ribose-1-phosphate; ADA, adenosine deaminase; AdoK, adenosine kinase; PRT, phosphoribosyltransferase; HGPRT, hypxanthine/guanine PRT; HXGPRT, hypoxantine/xanthine/guanine PRT; Ade, adenine; Gua, guanine; Xan, xanthine; Hx, hypoxanthine; Ado, adenosine, Guo, guanosine; Xao, xanthosine; Ino, inosine; 8-azaGua, 8-azaguanine (analogously shortened other 8-azapurines and 8-azanucleosides); 8-azaDaPur, 2,6-diamino-8-azapurine (2-amino-8-azaAde); Zaprinast, 2-(2-propoxyphenyl)-8-azaHx; Formycin, Formycin A (8-aza-9-deazaAdo); 8-azatheophylline, N(1),N(3)-dimethyl-8-azaXan; ESPT, excited state proton transfer

structure and molecular interactions.

8-azaGua was the first purine analogue demonstrated to exhibit antitumour activity.[30,31] It was long ago isolated, in crystalline form, from fermentation broths of *Streptomyces albus* var. *pathocidicus*, and its possible mode of biosynthesis examined by Hirasawa and Isono.[32]

There are now many reports on 8-azapurine analogues as modulators of biological targets, e.g. acyclonucleotides of 8-azapurines have been listed as potent antiviral agents,[41,42] and various derivatives as adenosine receptor antagonists.[43] A C(2)-substituted 8-azaHx (Zaprinast), first identified as an anti-allergic drug,[44,45] has been shown to be a selective inhibitor of phosphodiesterase 5,[46] leading to the finding of its ability to correct for erectile dysfunction, and stimulating the development of more effective drugs, such as Sildenafil (Viagra) and others.[47] And 8-azaGua is a useful probe for the ionic state of catalytic Gua residues in structured RNAs, such as ribozymes,[8,13] as well as in synthetic DNA fragments.[14,15]

The present review describes the fluorescence emission properties of 8-azapurines, their nucleosides and nucleotides, together with some structurally related isosteric analogues such as the 8-aza-9-deaza- and 8-aza-7-deaza- purines, i.e. pyrazolo[4,3-d]pyrimidines and pyrazolo[3,4-d]pyrimidines, respectively (Fig. 1), with particular emphasis on their tautomerism and acid-base properties, both in ground and excited states, and their widespread utility as substrates and/or inhibitors in many enzymatic reactions, and their potential as fluorescence probes in various biochemical systems.

2 Fluorescence emission of 8-azapurines and nucleosides

2.1 Fluorescence of 8-azapurines

It is well known that the canonical purines, pyrimidines and the corresponding nucleosides are characterized by very low fluorescence yields and short excited-state (S_1) decay times.[48,49] By contrast, the analogous 8-azapurines and their nucleosides display measurable fluorescence in neutral aqueous medium,[2] of obvious utility in studies on protein-ligand interactions, as well as in enzymology, including catalytic RNAs (ribozymes). Similarly, moderate to high fluorescence was reported for pyrazolopyrimidine analogs, the best known being Formycin A (8-aza-9-deazaadenosine[18,50]), which has found many applications in structural and analytical biochemistry.[51–56]

8-azapurines, and the corresponding nucleosides, may exist in various forms, including ionic and tautomeric structures. These usually differ markedly in their spectral properties, including fluorescence emission (see Tables 1 and 2), especially the pH-dependance of the emission of some of these, which has been profited from, e.g. to elucidate the mechanism of some catalytic RNAs (see below). To gain maximum information from fluorescence data, it is necessary to identify and characterize all fluorescent species of the fluorophores, including ionic and tautomeric forms, and identify those, which under physiological conditions, are reponsible for the observed fluorescence. We herein review the emission properties of free 8-azapurine bases and their alkyl deivatives, excluding the C(6)-unsubstituted 8-azapurines, which are known to undergo covalent hydration in aqueous medium.[1]

2.1.1 Overview. The room-temperature fluorescence of 8-azaGua (see Table 1) was long ago noted by Drobnik and Augenstein,[57] with the marked discrepancy between absorption and fluorescence excitation spectra of the neutral 8-azaGua erroneously ascribed to keto-enol tautomerism. Strong fluorescence of N(8)-methyl-8-azaGua was also noted by Albert and Taguchi.[37] Subsequently, a detailed analysis of the fluorescence excitation spectra of 8-azaGua and its derivatives methylated on the triazole ring[2,3] led to interpretation of the anomalous 8-azaGua fluorescence excitation as resulting from annular prototropic tautomerism,[2] most likely N(9)-H↔N(8)-H, but with possible participation of the N(7)-H protomer (see Section 2.1.2).

The most intensely fluorescent 8-azapurine reported is 8-azaDaPur (2-amino-8-azaAde), with quantum yield reaching 0.4, and increasing to 0.9 for the nucleoside,[58,59] with maximum emission at ca. 365 nm. These emission paramaters are comparable to those of 2-aminopurine,[18] a frequently used probe in nucleic acid and enzymological research.[22,29]

Most unexpected was the discovery of the intense fluorescence of 8-azaxanthine (8-azaXan) in weakly acidic aqueous medium.[6] This emission, with maximum at 420 nm, is characterized by an unusually large Stokes shift ($\sim$16000 cm^{-1}) and was subjected to detailed analysis, which led to identification of the emitting species as a phototautomeric monoanion (see below). In neutral aqueous medium (pH 7) this fluorescence disappears, due to dissociation of the triazole proton (pK_a 4.9, see Section 2.1.3). Various N-methyl derivatives of 8-azaXan are intensely fluorescent (see next Section).

Another example of phototautomerism is 8-azaisoGua, which, in its neutral form, reveals two-band fluorescence.[9] Similar, but more intense emission was recorded for the N(8)-methyl derivative. By contrast, the N(9)-alkyl derivative (and nucleoside) reveal single-band fluorescence, centered at 360 nm, recently applied to polynucleotide studies (Seela et al.[15,60]).

With the exception of 8-azahypoxanthine (8-azaHx), 8-azpurine analogs reveal moderate to intense fluorescence, as do the corresponding nucleosides (see Section 2.2). This fluorescence is higly sensitive to pH, solvent, and sometimes even isotope changes, suitable for biophysical applications.

Table 1 Spectral parameters for neutral and ionic forms of selected 8-azapurines in aqueous medium. UV spectral data are compiled in part from Albert,[1,33–35] Albert and Tratt,[36] Albert and Taguchi,[37] Nubel and Pfleiderer,[38] Blank et al.,[39] Islam and Nagamatsu,[40]. Unless otherwise indicated, fluorescence data refer to excitation at 280 nm. Unpublished data are indicated by an asterisk (*).

		Form	UV absorption		Fluorescence[b]		
Compound	pK_a	(pH)[a]	λ_{max} [nm]	ε_{max} [$M^{-1}cm^{-1}$]	λ_{max} [nm]	ϕ	τ [ns]
8-azapurine	2.05[c],4.84	n	263	7400	nf	<0.001	-
		ma	268	7800	nf	-	-
		cat(hydr)	248	8100	nf	-	-
8-azaAde	2.7,6.24	n(4.0)	273	10500	345	0.008	-
		ma(9.0)	275	10800	328	0.002	-
8-azaGua	1.2,6.5,10.8	n(4.5)	249	11200	395	0.05–0.33[c]	6.2*
		ma(8.9)	278	6200	355	0.03	6.6*
		da(12.2)	280	7900	360	0.12	-
8-azaHx	5.2,10.8	n(3.5)	253	8710	340	∼0.003	-
		ma(7.5)	273	9120	360	∼0.001	-
		da(12.2)	270	10100	nf	<0.001	-
2-(2-propoxyphenyl)-8-azaHx (Zaprinast)	5.1,∼10	n(3.0)	267	10700	400	∼0.02	-
		ma(7.0)	270	10900	-	-	-
8-azaDaPur	3.8,7.7	n(6.5)	280	8500	365	0.40	7.5,0.25
		ma(10.0)	290	6400	372	0.36	3.4
		cat(2.0)	253	9500	410	0.26	1.3, 8.4
8-azaIsoGua	2.2,5.4,∼12	n(4.3)	281	11000	360,420	∼0.017	0.3,8
		ma(7.5)	278	13400	360	∼0.005	<0.2
		cat(∼1)	277	9030	420	∼0.21	∼7
8-azaXan	4.96,10.2 4.66,9.79[e]	n(3.0)	263	6500	420	∼0.21[d]	9.0
		ma(7.0)	265	8500	nf	<0.001	-
		da(12.3)	285	6500	nf	<0.005	-

[a] n, neutral form; cat, cation; ma, monoanion; da, dianion; [b] nf, no detectable fluorescence in aqueous medium; nd, no data; [c] Value perturbed by covalent hydration; [d] Dependent on excitation wavelength. For 8-azaGua, the value 0.33 was determined with λ_{exc} 315 nm, and 0.05 with λ_{exc} 270 nm[2]; [e] Data from Nubel and Pfleiderer,[38] pK_a values determined electrochemically; [f] Data for 9-benzyl derivative[6]

Table 2 Spectral parameters for neutral and ionic forms of some N-methyl derivatives of 8-azapurines in aqueous medium. UV spectral data are compiled in part from Albert,[1,33–35] Albert and Tratt,[36] Albert and Taguchi,[37] Nubel and Pfleiderer,[38] Blank et al.,[39] Islam and Nagamatsu,[40]. Unless otherwise indicated, fluorescence data refer to excitation at 280 nm. Unpublished data are indicated by an asterisk (*).

		Form	UV absorption		Fluorescence[b]		
Compound	pK_a	(pH)[a]	λ_{max} [nm]	ε_{max} [$M^{-1}cm^{-1}$]	λ_{max} [nm]	ϕ	τ [ns]
N(9)-methyl-8-azapurine	0.3	n	264	7590	nd	-	-
		cat	263	5370	nd	-	-
N(8)-methyl-8-azapurine	3.2[c]	n	270	11480	nd	-	-
		cat(hydr)	254	10000	nd	-	-
N(9)-methyl-8-azaAde	2.8	n	277	10230	350*	∼0.02*	-
		cat	264	10700	nf	-	-
N(8)-methyl-8-azaAde	3.54	n	291	10700	380*	∼0.04*	-
		cat	281	11500	nf	-	-
N(7)-methyl-8-azaAde	2.45	n	285	9330	nd	-	-
		cat	284	11200	nd	-	-
N(9)-methyl-8-azaGua	8.1	n	251	11500	∼350*	<0.01*	0.16*
		ma	277	10000	362*	0.85*	6.7*
N(8)-methyl-8-azaGua	8.6, 1.86	n	293	7400	400	0.51	-
		ma	297	7900	410	0.45	-
N(7)-methyl-8-azaGua	8.25, 1.6	n	297	5100	390	0.09	-
		ma	300	5900	405	0.07	-
N(8)-methyl-8-aza-2,6-diaminopurine	4.85	n(7)	307	8100	412	0.88	12.7
N(8)-methyl-8-azaIsoGua	3.6,10.0	n(7)	284	∼7500	360,420	0.17	0.5,10
		cat(2)	287	∼7000	422	0.58	10.3
N(3)-methyl-8-azaIsoGua	2.3,5.6	n(4.3)	284	nd	368	0.013	0.4
		ma(9)	282	nd	369	0.10	1.4
N(9)-methyl-8-azaXan	5.36[e]	n(2.0)	255	9100	365,430[f]	0.075[f]	-
		ma(7.0)	278	7600	360[f]	0.06[f]	-
N(8)-methyl-8-azaXan	7.04[e]	n(4.0)	272	8700	420	0.50	12
		ma(11.0)	299	6450	420	0.60	12
N(7)-methyl-8-azaXan	7.22[e]	n(3)	273	6030	400,430	0.13	4.3
		ma(11.3)	304,302[b]	5750	401	0.23	4.9
N(1),N(3)-dimethyl-8-azaXan	4.47[e]	n(2)	271	6300	355	0.18[d]	3.7
		ma(7)	269	8500	nf	<0.001	-
N(1),N(3),N(7)-trimethyl-8-azaXan		n	280	7800	350	0.014	∼0.15*
N(1),N(3),N(8)-trimethyl-8-azaXan		n	276	10000	355	0.44	3.5

[a] n, neutral form; cat, cation; ma, monoanion; da, dianion; [b] nf, no detectable fluorescence in aqueous medium; nd, no data; [c] Value perturbed by covalent hydration; [d] Dependent on excitation wavelength. For 8-azaGua, the value 0.33 was determined with λ_{exc} 315 nm, and 0.05 with λ_{exc} 270 nm[2]; [e] Data from Nubel and Pfleiderer,[38] pK_a values determined electrochemically; [f] Data for 9-benzyl derivative[6]

2.1.2 Identification of emitting species. Ground-state tautomerism of 8-azapurines is more complex than that of the parent purines, due to presence of the ring nitrogen [N(8)]. Identification of the forms predominant in the ground state is therefore not easy, and it should be kept in mind that these may not necessarily be identical with the forms responsible for the observed fluorescence. The standard approach to resolve this problem is via examination of N-alkyl derivatives.

Amino and keto strukctures predominate in most amino and oxo 8-azapurine derivatives, analogously as in the purine series.[61,62] Such conclusions can be drawn from crystyallographic data, available for 8-azaAde hydrochloride,[63] its N(7)-methyl derivative[64] and 8-azaAdo,[65] and 8-azaGua and 8-azaHx derivatives.[63,66–68] Spectroscopic (FT/IR) studies of 8-azapurines also point to predominance of the keto-amino structures.[36,69]

Crystal structures show the presence of the N(9)-H in 8-azaGua,[66] while 8-azaGua hydrobromide crystals show the N(8)-H, N(3)-H structure.[68] The N(9)-H form was also observed in Zaprinast[45] but only the N(8)-H in 8-azaHx[67] and 8-azaXan.[70] The N(8)-H tautomer is found also in crystals of 1,3-dimethyl-8-azaXan (8-azatheophylline).[71] The latter compound has also been investigated using ^{15}N NMR in DMSO,[72] indicating 8:2 predominance of the N(8)H over the N(7)H tautomer, with the N(9)H form undetectable.

Fluorescence studies of N-alkyl derivatives (see Table 2) can not only identify the most fluorescent forms, but also help to resolve the problem of ground-state tautomerism. A good example is 8-azaGua, where there is a marked difference between various N-methyl derivatives in terms of emission and UV-absorption spectra.[2,3] While the N(9)-methyl derivative, with maximum absorption at 251 nm, is only weakly fluorescent, N(8)-methyl-8-azaGua reveals intense fluorescence (ϕ ~0.5, λ_{max} 400 nm), and UV absorption maximum at 293 nm. The UV absorption of 8-azaGua at pH~4.5 (neutral form) is clearly similar to that of the N(9)-methyl derivative (λ_{max} 249 nm), indicating predominance of the N(9)H tautomer, but its emission, with λ_{max} at 395 nm and excitation maximum at 290 nm,[2] must originate from the minor (~5% molar ratio), but strongly fluorescent, N(8)H form.

Similar behaviour is shown by 8-azaisoGua, which exists as a neutral species at pH~4,[9] the UV spectrum of which (λ_{max} 281 nm) is similar to that of the N(9)-alkyl derivative (Medza, unpublished), as well as to the nucleoside,[60] while the fluorescence excitation, showing double maxima at 284 and 254 nm, resembles that of the N(8)-methyl derivative, the latter showing good agreement between UV absoption and fluorescence excitation spectra. It was concluded that the fluorescence of 8-azaisoGua, and also its 3-methyl derivative, are due mainly to the minor N(8)H protomer, the major form being N(9)H.

Predominance of the highly fluorescent N(8)H tautomer in 8-azaXan is evident from a comparison of the emission properties of three N-alkyl derivatives, alkylated on the triazole ring. The most fluorescent of these, N(8)-methyl-8-azaXan, exhibits maximum emission at 420 nm in aqueous solution and 355 nm in non-protic media, and clearly resembles the fluorescence of 8-azaXan.[6] Much weaker fluorescence of the N(9)-benzyl derivative is centered near 365 nm in water, and its excitation maximum is close to 260 nm, quite different from that of 8-azaXan (275 nm), so that the N(9)H tautomer must be reagrded as undetectable by the fuorescence method. By contrast, participation of the N(7)H form in the ground state of 8-azaXan cannot be excluded, as shown by the spectral properties of the N(7)-methyl-8-azaXan (Table 2).

No traces of the N(8)H tautomer can be detected, using this method, in the fluorescence of the neutral 8-azaDaPur.[58] The N(8)-methyl derivative of the latter compound is intensely fluorescent (Table 2), but its emission maximum (410 nm) differs significanly from that of the parent compound (365 nm). Moreover, the emission of the neutral 8-azaDaPur is virtually excitation-independent. The N(9)-alkyl derivative emitts at ~368 nm,[58] so the emission of 8-azaDaPur originates from the predominant N(9)H form.

For 8-azaAde, which is weakly fluorescent at 345 nm, the emission seems to originate from the major N(9)H form, since the spectral parameters of N(8)-methyl derivative are quite distant from those of 8-azaAde (Table 2). Hence emission data do not confirm previous theoretical speculations of the predominance of N(8)H form in 8-azaAde, at least in aqueous medium.

One of the important applications of the isosteric nucleobase analogs, and their N-alkyl derivatives, are spectral investigations of the enzyme-ligand complexes.[4,51] The ligands participating in the complexes may adopt different ionic/tautomeric forms than those predominating in solution. Thus, detailed knowledge of the spectral properties of various forms is necassary for proper interpretation of the data.

2.1.3 The pH-dependence. The pH-dependence of 8-azapurine fluorescence is determined by the ground-state acidity of the triazole proton, the basicity of the pyrimidine nitrogens, and in the case of the oxo-substituted compound, additionally by the acidity of the pyrimidine proton(s).

8-azapurines are much more acidic and less basic than the corresponding parent purines[1] (see also Fig. 2). It is a general rule for 8-azpurines that the triazole proton is more acidic than the analogous imidazole proton in purines, sometimes by as much as ~5 pK units,[1] and as a consequence at pH 7 many 8-azapurines exist exclusively, or predominantly, in the anionic forms. This includes 8-azaAde (pK_a 6.1), 8-azaGua (pK_a 6.5), 8-azaHx (pK_a 5.1) and 8-azaXan (pK_a 4.9), with the only exception of 8-azaDaPur (pK_a 7.7). By contrast, the acidity of the pyrimidine proton(s) in oxo-8-azapurines is only slightly (~1 pK unit) enhanced relative to the analogous

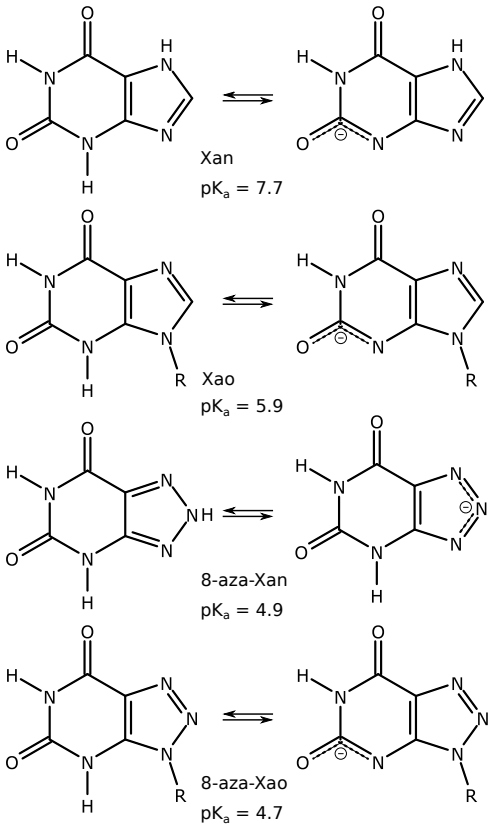

Fig. 2 Ionization of Xan, Xao, 8-azaXan, and 8-azaXao (R = ribose). Note that the neutral form of 8-azaXan is shown as the N(8)-H protomer. In DMSO, the 1,3-dimethyl derivative (8-azatheophylline) consists of an equilibrium mixture of the N(8)-H and N(7)-H forms in the proportion 8:2,[72] and uniquely as the N(8)-H in the crystal.[71]

purine species, as shown by titration of N-alkyl derivatives. As a consequence, in all known oxo-8-azapurines the triazole proton is more acidic that the pyrimidine one. The basicity of the pyrimidine nitrogens in 8-azpurines is lowered relative to that of the corresponding purines, as illustrated by 8-azaAde (pK_a 2.1) vs. adenine (pK_a 3.5).

Fluorimetric titration curves give pK_a values virtually identical to those obtained by spectrophotometric procedures (Wierzchowski), but does not indicate identity of ground- and excited-state acidities (see below). The protonated and deprotonated forms of 8-azaAde and 8-azaGua are only weakly fluorescent (Table 1), and no emission was detected for the anionic forms of 8-azaHx and 8-azaXan. Only for 8-azaDaPur was intense emission of the ionic form reported.[58]

Quite different behaviour is observed for N-methyl derivatives of the oxo-8-azapurines (Table 2), especially those methylated on the triazole ring: high fluorescence yields were reported for the monoanionic forms of N-alkyl derivatives of 8-azaGua,[2,4] 8-azaisoGua[9] and 8-azaXan.[6,10] This difference

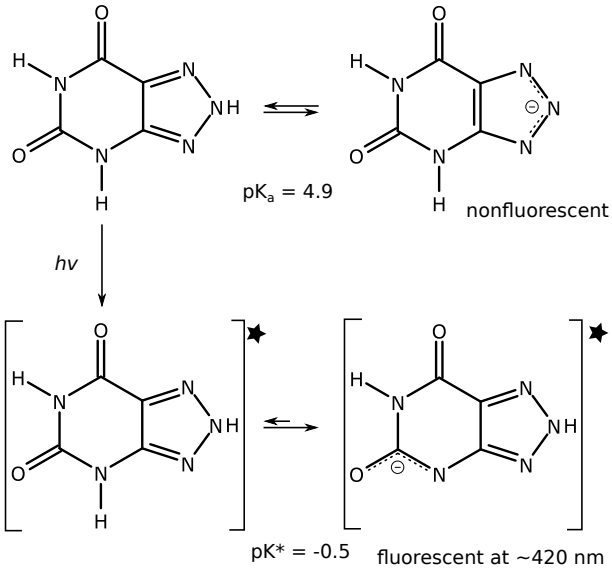

Fig. 3 Proposed interpretation of 8-azaXan blue fluorescence in aqueous medium(pH<6). Note that the last step, deprotonation of N(3), is very rapid (∼10 ps).

is due to the fact that anions of unsubstituted 8-azapurines are generated by dissociation of the triazole proton, while those of N-methyl derivatives of oxo-8-azapurines by dissociation from the pyrimidine ring. It should be stressed that the N-methyl derivatives are generally much less acidic than the parent 8-azapurines (Table 1 and 2).

Thanks to marked differences between the emission form neutral and ionic forms, 8-azapurines can be applied to investigations of the enzymatic processes, especially those where the ionic intermediates are postulated to exist in some stages of the reaction. Some examples of such studies are available in the literature (see next chapter).

2.1.4 Excited-state proton transfer. Species responsible for fluorescence emission may not necessarily be the same as those responsible for absorption, due to possibility of excited-state proton transfer.

Excited-state proton transfer (ESPT) is a fairly common phenomenon among fluorescent N-heterocycles,[73] including fluorophores of biological interest, like lumazine, luciferin and the fluorophore of green fluorescent protein.[74,75] This phenomenon is manifested typically as a solvent-, isotope- and buffer ion-dependent dual fluorescence, and/or particularly large Stokes shift of the observed single emission band. The high sensitivity of the emission parameters to the environment is particularly useful for applications in enzymology.[76–78]

Thermodynamic possibility of ESPT is a consequence of the large differences between acid-base properties of ground vs. excited states. These differences may be calculated from spectral data using the Foerster cycle,[79,80] and may be as large

as 7-8 pH units.[73,81] Proton transfer rates are typically comparable to, and sometimes exceed, those of the radiative excited-states decay.[81]

Examples of ESPT among purine and purine nucleoside analogues have been briefly reviewed recently,[82,83] some referring to the 8-azapurines and their N-alkyl derivatives. In particular, 8-azaXan in slightly acidified 1% aqueous methanol exhibits two fluorescence bands, with maxima at ∼335 nm and ∼420 nm, whereas in water (pH <4.5) only the long-wavelength band is present, resulting in a large Stokes shift, 16000 cm^{-1}.[6] Comparison with the N(8)-methyl derivative, showing similar emission at pH 2-12, allowed identification of the 420 nm fluorescence band in 8-azaXan as a phototautomeric anion, formed in the excited state of the fluorescent N(8)-H protomer, as a result of ESPT from N(3), as depicted in Fig. 3. Using spectral parameters of N(8)-methyl-8-azaXan, the $pK^{\star}$ of the N(3)-H in the excited state was calculated to be approximately -0.5,[6] pointing to rapid deprotonation, even in moderately acidic aqueous medium. This process is much slower in alcohols, resulting in appearance of emission from the neutral species at ∼340 nm. The fluorescent anionic form is absent in the ground state of 8-azaXan, since, as in most 8-azapurines, the triazole proton in the ground-state is more acidic than the N(3)-H (see above). As expected, only the short-wavelenghth emission band (355 nm) is present in the fluorescence of 8-azatheophylline (1,3-dimethyl-8-azaXan, see Table 2).

Solvent- and isotope-dependent dual fluorescence is also observed for neutral forms of 8-azaisoGua and its N-methyl derivatives, particularly N(8)-methyl-8-azaisoGua.[9,82] In the latter compound, dual emission is observed in neutral aqueous medium, and the intensities of the two bands, with maxima at ∼360 and ∼430 nm, are markedly dependent on buffer ion concentration, but independent of excitation wavelength, also indicative of intermolecular excited-state proton transfer.[50] The protonated form of 8-aza-isoGua (pK_a ∼2.2) also exhibits the long-wavelength fluorescence band (420 nm), with a large (∼12000 cm^{-1}) Stokes shift, absent in the cations of N(3)-alkylated derivatives,[9] suggesting that excited-state N(3)-H deprotonation is responsible for the dual emission observed in 8-aza-isoGua.

Another example of ESPT is observed in 8-azaDaPur, but only in weakly acidic conditions[58] where dual emission is observed, with a long-wavelength band, centered at ∼410 nm, showing a Stokes shift of >11000 cm^{-1}. Comparison with the N(8)-methyl derivative identifies the 410 nm band as originating from the neutral N(8)H tautomer, generated by excited-state deprotonation of the cation. The $pK^{\star}$ for this process was estimated to be less than -2.[58] In the ground state, this tautomer is undetectable, and the phototautomeric process in the neutral aqueous medium is too slow to compete with the excited-state decay of the dominant tautomer, N(9)H. However, dual emission can be observed in non-aqueous medium, like isopropanol, containing ∼0.1 M acetic acid-triethylamine mixture (10:9), the latter acting as a catalyst for the presumed two-step proton transfer reaction.

The excited-state proton transfer rate is highly dependent on the microenvironment, particularly the hydrogen-bond network, and for this reason nucleobase and nucleoside analogs undergoing ESPT can be used as sensitive probes to study their interactions with various biomolecules, including enzymes and ribozymes.

2.2 Fluorescence of 8-azpurine nucleosides

Nucleosides and nucleotides of 8-azapurines usually differ markedly, in terms of spectral and acid-base properties, from the corresponding nucleobase analogs, because the latter display annular tautomerism, not possible in N-ribosides. This difference is analytically important, since it allows sensitive measurement of the activity of various glycolytic enzymes by means of fluorescence spectroscopy.[3,84] Fluorescence parameters of 8-azapurine ribosides are summarized in Table 3. In general, they are qualitatively similar to those of the N(9)-alkyl derivatives of the corresponding 8-azapurines. Spectral characteristics of the deoxyribosides are nearly identical to those of the corresponding ribosides.[2,7,85,86]

2.2.1 8-Azaguanosine and 8-azaadenosine. 8-Azaguanosine (8-azaGuo) and 8-azaadenosine (8-azaAdo) phosphates can substitute for the parent purine nucleotides in catalytic RNAs, without losing biochemical activities, and thanks to this they can serve as fluorescent probes of the catalytic mechanism.[13,16] 8-AzaGuo exhibits instense fluorescence in the deprotonated form (pK_a ∼9), and very weak as a neutral species. By contrast, 8-azaAdo is moderately fluorescent in neutral medium, but not upon protonation (Table 3). Their applications include determination of the transition-state structure in ribozyme-catalyzed reactions (see Section 3.5).

2.2.2 8-Azainosine and 8-azaxanthosine. Unlike 8-azaHx, 8-azainosine is moderately fluorescent in neutral aqueous medium, but this fluorescence is due to the anionic form of the compound and disappears at pH<7.[2,7] The emission of 8-azaxanthosine, centered at ∼365 nm, can be observed over a broad pH area, but this emission is due to the monoanionic form (pK_a 4.6), which at the pH<pK_a is generated by ESPT.[10] The very low acidic pK_a value of this compound is analogous to that obesrved in xanthosine (Kulikowska et al.,[52] see Figure 2), and is a consequence of the fact that the N(9)H tautomer is energetically much less favored than N(7)H in xanthine and N(8)H in 8-azaXan.

2.2.3 Other nucleosides. The 8-azaDaPur nucleoside belongs to the most intensely emitting nucleosides known, with

Table 3 Spectral parameters for neutral and ionic forms of selected 8-azapurine nucleosides. The UV spectral data are compiled from Davoll,[87] Hutzenlaub et al.,[88] Elliott and Montgomery,[89,90] Seela et al.,[7] and Jiang and Seela.[15]

		Form	UV absorption		Fluorescence[b]		
Compound	pK_a	(pH)[a]	λ_{max} [nm]	ε_{max} [$M^{-1}cm^{-1}$]	λ_{max} [nm]	ϕ	τ [ns]
8-azapurine riboside	<1	n(1)	262	6600	nf	<0.001	-
(8-azanebularine)		n(11)	261	6300	nf	<0.001	-
8-azaAdo	2.2	n(7.0)	278	12000	352	0.068	0.8
		c(1.0)	262	12300	nf	-	-
8-azaGuo	8.05	n(6.0)	256	12900	347	<0.01	~0.1
		ma(10.2)	278	11700	360	0.55	5.6
8-azaIno	7.9	n(5.0)	254	10000	nf	<0.001	-
		ma(11.0)	275	10700	357	.018	-
8-aza-deoxy-IsoGuo	8.4	n	251,285	6060	~365	<0.05	nd
		ma	nd	nd	370	>0.2	nd
8-azaXao	4.6	n(3.0)	255	8600	362,430	~0.07	0.8,5.3
		ma(8.0)	278	10000	362	~0.11	0.9
8-aza-2,6-diamino-purine riboside	~3.2	n(7)	286	10800	367	~0.9	nd

[a] n, neutral form; cat, cation; ma, monoanion; da, dianion; [b] nf, no detectable fluorescence in aqueous medium; nd, no data.

fluorescence yield ~0.9.[59,84] Since the corresponding purine nucleoside is used as a probe in biological research,[18,22] it is to be expected that this ca. 100-fold stronger emitting analog can better serve this purpose.

Recently Seela et al.[15,60] found that the moderate fluorescence of 8-azaisoGuo and the corresponding nucleotide is not quenched upon incorporation into oligomers, as frequently found for other nucleosides. This led to interesting applications in the area of polynucleotide interactions and assemblies.[15]

2.2.4 N7- and N8-ribosides. It has been demonstrated recently that the non-typical nucleosides, like N7-β-D-riboside and N8-β-D-ribosides of various purine analogs can be enzymatically generated using wild or modified enzymes of nucleoside metabolism[59,91] and some of them can be also degraded by these enzymes,[84] which makes them applicable to analytical or mechanistic biochemistry. These non-typical nucleosides differ spectrally from the natural N9-β-D-ribosides, frequently showing higher fluorescence yields and red-shifted spectra, much more suitable for analytical research. Some examples include very highly fluorescent N8-β-D-ribosyl-8-azaXan, generated by xanthosine phosphorylase in the reverse reaction[6] and non-typical ribosides of 8-azaDaPur, which are selective substrates for various types of purine-nucleoside phosphorylase (PNP).[84]

3 Biochemical aspects and applications

3.1 Metabolism and cytotoxicity of 8-azapurines

Early interest in 8-azapurine metabolism[92] was further stimulated by the discovery of anti-cancer activities of some of these, particularly 8-azaGua, 8-azaAdo and 8-azaIno, summarized by Montgomery[93] and Albert,[1] and reviewed by Robins and Revankar.[94] The overall mechanisms of the cytotoxicity of 8-azapurines require further characterization, but some general conclusions can be formulated, as follows.

Transformation of 8-azapurines, and/or their nucleosides, to their 5'-nucleotides is a key step in their metabolic activation via the purine salvage pathway: (a) phosphoribosyltransferases (PRTase, e.g., hypoxanthine-guanine PRTase - HGPRT, adenine PRTase - APRT) in the case of 8-azaGua, 8-azaHx, and 8-azaAde, or (b) purine nucleoside phosphorylase (PNP), followed by nucleoside kinase (typically AdoK), in the case of some 8-azapurines, like 8-azaHx and 6-methyl-8-azaHx.[93,94] Neither pathway is very effective in mammalian systems, since both 8-azaGua and 8-azaHx are only weak substrates for mammalian, including human, HGPRT,[95] and 8-azaHx is a very poor substrate for the reverse (synthetic) pathway of PNP (see Section 3.2.2). There is one report implicating the PNP-nucleoside kinase pathway in the activation of 8-azaGua in mammalian tissues.[96] However, cellular resistance to 8-azapurines, including 8-azaGua, is typically associated with lack of PRTase activity.[93,97]

The metabolic activation of nucleosides, especially 8-azaAdo and 8-azaIno, is simpler, since both compounds readily respond to Ado kinase,[98,99] and this is correlated with rel-

atively strong cytotoxicity.[94] Similarly, lack of cytotoxic effects of 8-azaXan can be explained by resistance to both potential activating pathways. 8-azaGuo is less toxic than 8-azaGua, due to the absence of Guo kinase activity in mammalian cells.[100] Nevertheless, there are reports[93] that, in some cell strains, 8-azaGuo is almost as toxic as 8-azaGua. This could be due to slow nucleoside cleavage by PNP and/or nucleoside hydrolases (see Section 3.2). Partial resistance to 8-azapurines may also result from high activity of catabolic enzymes, like Guanase or 5'-nucleotidase.[101]

Once in the form of the 5'-nucleotide, these can be further phosphorylated by nucleotide kinases, and the resultant triphosphates incorporated into RNA,[93] leading to toxic effects. Interestingly, 8-azaIMP is metabolically transformed and incorporated as 8-azaAMP or 8-azaGMP.[102] However, Chen and Sheppard[103] have shown that incorporation of 8-azapurine nucleotides into RNA may not always be efficient, since, e.g., 8-azaATP is a suicidal substrate for the yeast poly(A) polymerase. By contrast, 8-azaGTP is apparently a fairly good substrate for RNA polymerases from various sources, both in vitro and in vivo,[8] although it cannot initiate translation.[104–106] In tRNA, 8-azaGua can replace Gua via the tRNA-guanine ribosyltransferase reaction.[107]

5'-Nucleotides of 8-azapurines can also be metabolized by enzymes of purine metabolism, e.g., 8-azaIMP is readily oxidized to 8-azaXMP by IMP dehydrogenase,[108] and the latter is a moderately good substrate for GMP synthetase.[109] It should also be noted that 8-azaXMP is also a relatively good inhibitor of OMP decarboxylase ($K_i \sim 0.4\ \mu M$), a key enzyme in pyrimidine biosynthesis.[110] Differences in metabolism of 8-azapurines, observed in some parasites, e.g., *Leishmania* and *Plasmodium*, are pharmacologically important.[111,112]

8-azapurines are probably not incorporated into DNA in vivo, but some exceptions have been noted in bacterial systems,[92] and 8-azaGua metabolites were long ago reported as inhibitors of ribonucleotide reductase.[113,114] They are not mutagenic, a necessary condition for utilization of 8-azaGua as a selective growth inhibitor in standard mutagenicity tests, which are based on mutations in the HGPRT gene, with resultant resistance to 8-azaGua.[115–117] 2-Deoxynucleosides of 8-azaAde are only weakly cytotoxic, whereas the respective 5'-bis(pivaloyloxymethyl)-deoxynucleotide derivatives, which are 8-azaAde pro-drugs, display significant toxicity, possibly due to reported inhibition of DNA synthesis by the triphosphates, although, surprisingly, these triphosphates undergo incorporation into DNA *via* polymerase α *in vitro*.[118] Cytotoxicity of 8-azapurines may also be related to the activity of some analogs as receptor agonists or antagonists, recently reviewed by Giorgi and Scartoni.[43]

3.2 Interactions of 8-azapurines and their nucleosides with enzymes of purine metabolism

3.2.1 Nucleosidases (nucleoside hydrolases). Nucleoside hydrolases are glycosidases that catalyze the irreversible hydrolysis of the N-glycosidic bond of β-ribonucleosides to liberate ribose and the free purine or pyrimidine base, as follows:

$$\beta - \text{ribonucleoside} + H_2O \longrightarrow \text{base} + \text{ribose}$$

These enzymes are characterized by a stringent specificity for the ribose moiety, but exhibit broad variability in their preference for the purine or pyrimidine base. They are very widely distributed in bacteria, yeast, protozoa, mesozoa, insects, the genes of which all exhibit a recurring N-terminal motif DXDXXXDD, also present in plants, amphibians and fish (for review, see Versees and Steyaert[119]). The metabolic role of these enzymes has been well established for several parasitic protozoa, in which they play a key role in the salvage pathway, which scavenges purines from the environment, allowing recycling of the free bases to nucleotides for DNA synthesis. Quite strikingly, neither nucleosidase activities, nor their encoding genes, have been detected in mammals, and thus represent attractive targets for drug development. There are scattered reports of 8-azapurine nucleosides as substrates of nucleosidases, but limited to only qualitive observations, and this is worhty of further study, especially with fluorescent analogues. We limit ourselves here to one exceptionally unique member of this family of enzymes, 5'-AMP nucleosidase.

3.2.2 5'-AMP Nucleosidase. There are now many reports on the existence of nucleotide N-hydrolases, which cleave the glycosidic bond of 5'-nucleotides to release the aglycone and ribose phosphate (Yang et al.,[120] Dupouy et al.,[121] and references therein). We here limit ourselves to one of the first reported, and an exceptionally unique member of this family of enzymes.

Hurwitz et al.[122] first reported the presence in *A. vinelandii* of an unusual enzyme analogous to a nucleosidase, in that it catalyzes the irreversible cleavage of 5'-AMP, but not adenosine, to adenine and ribose-5-phosphate, in the presence of MgATP, an allosteric activator of the enzyme, as follows:

$$\text{AMP} \xrightarrow{\text{MgATP}} \text{Adenine} + \text{ribose} - 5 - \text{phosphate}$$

Inorganic phosphate (or arsenate) appears to serve as an allosteric inhibitor via competitive inhibition relative to MgATP. The enzyme appeared to be quite specific for 5'-AMP, inasmuch as a broad range of nucleosides and nucleotides were not substrates (but see below).

Structural studies of the E. coli enzyme revealed that it is a homohexamer with 32-point symmetry, and that the catalytic

domain of each monomer resembles the overall topology and active site of members of the nucleoside phosphorylase family NP-1, which includes the trimeric and hexameric purine nucleoside phosphorylases (Zhang et al.,[123] and references cited).

In a study on the substrate and inhibitor specificity of the A. vinelandii enzyme, DeWolf et al.[124] reported that 2-aminoAMP, 8-azaAMP, 2-deoxyAMP, 3-deoxyAMPand NMN were weak substrates, with V_{max} values $<10\%$ that for AMP. However, a closer examination of their data (including their Table 2) reveals that 8-azaAMP, with a K_m of 85 μM, as compared to 120 μM for AMP, and a V_{max}/K_m of 0.76×10^{-1} min^{-1} as compared to 2.8×10^{-1} min^{-1} for AMP, is in fact a good substrate.

Furthermore, it should be noted that 8-azaAdo (hence also 8-azaAMP) is fluorescent, with an emission maximum at physiological pH at 352 nm and a quantum yield of 0.068 (Table 3). Under the same conditions, the emission maximum of 8-azaAde is displaced to 320 nm with a very low quantum yield of 0.002 (Table 1). It follows that 8-azaAMP should be an excellent substrate for continuous fluorimetric monitoring of AMP nucleosidase activity, as well as for studying its mode of interaction with the enzyme in solution. Current methods are based on chromatography to reveal release of free adenine, or measurement of formation of reducing sugar from AMP, both very inconvenient. The reaction has also been followed spectrophotometrically by monitoring the change in absorbance at 275 as AMP is cleaved to adenine,[124] but the change is small (1.6 absorbance units cm^{-1}), hence not very sensitive.

Potential inhibitors examined by DeWolf et al.[124] included a close structural isosteric analogue of AMP, 8-aza-9-deazaAMP with a non-cleavable C-C glycosidic bond, viz. Formycin-5'-phosphate (FMP). This proved to be a potent competitive inhibitor with respect to AMP, with a K_i of 43 nM, so that it binds 2600-fold more tightly to the enzyme than AMP. With the enzyme from E. coli, FMP binds 1200-fold more tightly than AMP.[125] Even Formycin itself is a reasonably good competitive inhibitor, with a K_i of 5 μM. The structural basis for this tight binding is therefore of considerable interest (see below).

The crystal structure of E. coli AMP nucleosidase in a complex with Formycin-5'-phosphate, reported by Zhang et al.,[123] displays schematically the mode of binding of the aglycone, pyrazolo[4,3-d]pyrimidine, as the N(8)-H protomer (Fig. 4). The authors concluded that Asp428 acts as a hydrogen bond donor to offset the partial negative charge and stabilize the transition state. The same Figure shows, for comparison, our proposed mode of binding of 8-azaAMP, which is virtually identical with that for Formycin-5'-phosphate, and explains why 8-azaAMP is a good substrate.

Surprisingly, no attention appears to have been paid to the

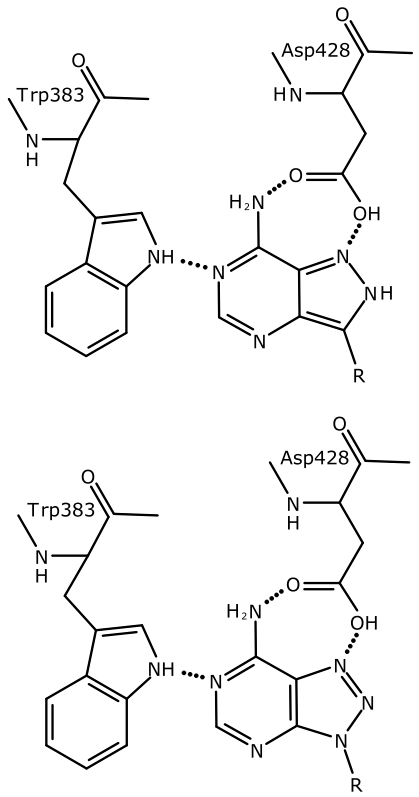

Fig. 4 Mode of binding by AMP nucleosidase of the aglycone of Formycin-5'phosphate (upper) and that proposed for 8-azaAMP (lower).

fact that both Formycin and its 5'-phosphate are highly fluorescent, and could be employed as probes for studying their mode of interaction with the enzyme in solution.

3.2.3 Substrate/inhibitor properties of 8-azapurines, and nucleosides, towards various forms of PNP. Purine nucleoside phosphorylases (PNP, E.C. 2.4.2.1) catalyze the reversible phosphorolysis of purine ribo- and 2-deoxyribonucleosides, as follows:

$$\beta-\text{purine}-\text{nucleoside}+\text{ortophosphate} \rightleftharpoons \text{purine base}+\alpha-\text{D}-\text{pentose}-1-\text{phosphate}$$

The mammalian trimeric forms of PNP are specific for (deoxy)guanosine and inosine, while some bacterial forms, e.g., the hexameric E. coli PNP-I, accept Ado and dAdo as well (see Bzowska et al.,[126] Pugmire and Ealick,[127] and Edwards[128] for reviews). Several other forms of PNP exist, notably the inducible xanthosine phosphorylase from *E. coli*, known also as PNP-II,[129] and the ubiquitous 5'-methylthioadenosine phosphorylase.[130]

PNP is regarded as a therapeutic target for many health disorders, including T-cell immunodeficiencies,[131] T-

cell leukemias,[132,133] psoriasis,[134] parasitic infections, e.g., malaria,[135,136] and cancer, e.g., gene-oriented cancer therapy, utilizing the so-called suicidal gene strategy.[137,138] Recent advances in understanding the mechanisms of PNP led to development of a series of transition-state analogues, with significant pharmacological potential (see Schramm[139]). In particular, the so-called immucillins, iminoribitol nucleoside analogues, mimicking the putative oxocarbenium ionic transition-state structure of the phosphorolytic process, are the most potent PNP inhibitors known to date.[132,139,140] Immucilin H (known also as Forodesine) is currently in clinical trials.[141–143]

Various forms of PNP have been exploited for the preparative synthesis of various nucleoside analogues, including 8-azanucleosides.[2,59,91,144,145]

PNP accepts a variety of nucleoside, and purine nucleoside analogues, as substrates (see Bzowska et al.,[126] for review). Although 8-azapurine nucleosides (8-azaGuo, 8-azaIno, 8-azaAdo) are known to be relatively resistant to phosphorolysis by both mammalian and bacterial forms of PNP (but see below), probably accounting for the much lower cytotoxicity of 8-azaGuo relative to 8-azaGua (cf. Parks and Agarwal,[146] Doskocil and Holy,[147]), some 8-azapurines are substrates for the reverse, synthetic pathway. Additionally, some 8-azapurine derivatives exhibit marked inhibitory activity towards various forms of PNP. Thanks to their intense fluorescence, 8-azapurine nucleosides may be exploited as fluorescent probes in mechanistic studies (see below), and as sensitive analytical tools for PNP activity assays.[3,84]

Early reports on 8-azapurines as substrates for PNP in the reverse synthetic pathway were reported for the mammalian enzyme by Parks and Agarwal[146] and for *E. coli* PNP by Doskocil and Holy.[147] PNP was subsequently postulated as an HGPRT-independent, but nucleoside kinase-dependent, pathway for metabolic activation of 8-azaGua in some organisms.[96] Until recently, these reactions were regarded as irreversible, but, in fact, very slow phosphorolysis of 8-azaGuo by calf PNP can be observed.[4]

Reports on the kinetics of 8-azapurine nucleoside synthesis catalyzed by calf and *E. coli* PNP-I as a function of pH[2,3], concluded that 8-azapurines are relatively poor substrates for the bacterial (*E. coli*) enzyme, with k_{cat} values in the range of 1% that of the natural purines. By contrast, mammalian (including human) forms of PNP quite effectively catalyze the synthesis of 8-azaGuo at pH$<$7. Slow synthesis of 8-azaIno was also observed, but only at pH$<$6.5. The marked pH-dependence of K_m in both cases suggests that only the neutral forms of 8-azaGua and 8-azaHx (i.e. those dominant in weakly acidic media, see Section 2.1) are accepted as substrates.[2]

Phosphorolysis of 8-azaGuo with calf PNP in 50 mM phosphate, pH 7, is ca. 2 orders of magnitude slower than phosphorolysis of Guo,[4] and, consequently, much slower than the reverse (synthetic) reaction in phosphate-free medium. 8-azaIno and 8-azaAdo were not detectably phosphorolyzed under the same conditions. The equilibrium constant for 8-azaGuo synthesis by the calf enzyme, is $\sim$320 at pH 7, compared to $\sim$50 for natural purine nucleosides; hence, in the presence of excess ribose-1-phosphate (R1P), the reaction proceeds virtually quantitatively in phosphate-free medium. This reaction has useful analytical application, thanks to the high fluorescence of the 8-azaGuo monoanion (observable at pH$\geq$7, see Section 2.2.4), and can be profited from for continuous monitoring of PNP activity in 2000-fold diluted blood lysates, using fluorimetric methods.[3] The same reaction may also be employed for a highly sensitive unique quantitative assay of R1P in cell lysates.[2] Previous methods for determination of intracellular levels of pentose phosphates were reviewed by Camici et al.[148]

3.2.4 Interactions of 8-azapurines, pyrazolopyrimidines and Formycins with PNPs: structural studies. Interactions of PNPs with substrates and inhibitors, including purine analogues, have been widely studied by a variety of theoretical and physicochemical methods, including molecular modeling, X-ray crystallography, NMR and fluorescence spectroscopy (see Ealick et al.,[149] Erion et al.,[150,151] Deng et al.,[152] Tebbe et al.,[153] Bzowska et al.,[11,126] Wielgus-Kutrowska et al.,[12] Afshar et al.[154]). These investigations were directed to elucidation of binding modes and reaction mechanisms, and with a view to development of novel and more selective inhibitors (e.g. Schramm,[132,139] Ho et al.[155]).

The mechanism of nucleoside phosphorolysis by PNP has been partially elucidated, on the basis of kinetic and crystallographic data, as proceeding via the oxocarbenium ion centered on the ribose oxygen-C(1) bond (see Fig. 5). Based on this mechanism, several transition-state analogues were synthesized, and found to exhibit picomolar association constants.[139,155] It is still being disputed as to how the transition state is realized, and, consequently, which tautomeric/ionic form of the purine is the true substrate for the reverse reaction. There are at least 3 possibilities, leading to three structures shown in Fig. 5. Experimental data suggest that the reverse reaction requires the purine substrate to be bound as the neutral species, with the imidazole proton either on N(7) or N(9) (see below).

Crystallographic structures of various forms of PNP have been determined (see Bzowska et al.,[126] Lewkowicz and Iribarren[158] for reviews). These include human PNP complexed with Gua, and with 8-azaGua (Fig. 6).[159] Interaction of both compounds with the active site is essentially similar, with the strongest hydrogen bond between N(1)-H and Glu201 (2.5 Å), characteristic also for Ino, Guo, Hx and the potent transition-state inhibitor immucillin H.[150,151,156] In contrast to the Gua-

Table 4 Kinetic parameters for enzymatic ribosylation of 8-azapurines by PNP from various sources, with ribose-1-phosphate as ribose donor. Data from Wierzchowski et al.[2,6]

Substrate	Enzyme source	pH	K_m [μM]	k_{cat} [relative]*
8-azaGua	Calf	7.0	101	∼20%
	Calf	8.05	680	∼19%
	E. coli-I	7.0	>200	<1%
	Human	7.0	225	
8-azaHx	Calf	7.0	>600	∼0.3%
	Calf	6.0	175	
8-azaAde	Calf	6–7		no reaction
	Human	7.0		no reaction
	E. coli-I	6.0	200	∼0.5%
8-azaXan	*E. coli*-II	5.5	175	
	E. coli-II	7.0	∼330	∼0.8%

*k_{cat} relative to that for the respective purine.

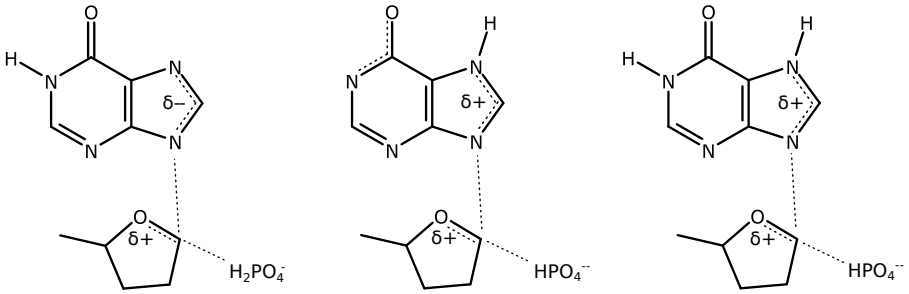

Fig. 5 Postulated structure of the transition state of PNP-catalyzed phosphorolysis of Ino proposed by Erion et al.,[150] (left), Tebbe et al.,[153] (middle), as modified by Edwards,[128] and Kicska et al.[156] (right). Note that, with exception of Erion model, the phosphate is in the dianionic form, as postulated by Edwards,[128] and confirmed by kinetic data.[157]

PNP complex, the 8-azaGua-PNP complex exhibits an additional hydrogen bond between N(8) and Thr242 (2.8 Å), and Asn243 binds to N(7) instead of O^6 in guanine (2.8 Å). In both structures there is also an intrinsic sulfate molecule, binding to N(9), but no indication of dominant tautomeric form(s) is discernible.

Several spectroscopic investigations were directed to elucidation of the role of tautomerism and ionic state of ligands bound to PNP (purine or purine analogue).[4,5,11,51,52,152,160] *E.coli* PNP-I binds Formycin A (a fluorescent C-nucleoside inhibitor) and some its methyl derivatives in a fixed, neutral tautomeric form. Furthermore, 8-azaGua forms a highly fluorescent complex with calf spleen PNP, the binding constant of which corresponds to the kinetically determined K_m ($\sim$100μM). Analysis of the fluorescence emission (λ_{max} $\sim$375 nm) and excitation spectra of this complex[4,5] excluded the major N(9)-H tautomer of 8-azaGua from the complex structure, and pointed to participation of either the N(7)-H or N(8)-H form (see Section 2.4). And ^{15}N NMR analysis of the human PNP-hypoxanthine complex clearly pointed to participation of the N(7)-H tautomer.[152]

Additional support for the foregoing was provided by analysis of a series of complexes of calf PNP with N(9)-phosphonoalkoxy-substituted purine analogues, including the highly fluorescent complexes with phosphonomethoxy(PME)-8-azaGua[4,5] and phosphonoalkoxy-8-aza-2,6-diaminopurine.[11] The phosphonoalkoxy analogues represent a class of bisubstrate analogue PNP inhibitors, binding very tightly to the enzyme in the absence of phosphate, and much less so in the presence of phosphate, due to competition for the binding site. The emission properties of the enzyme-bound PME-8-azaGua are quite distinct from those of bound 8-azaGua (blue shift $\sim$35 nm), indicating that the latter cannot be bound as the N(9)-H tautomer.[4]

The foregoing data clearly indicate that 8-azapurines and their derivatives, as well as analogous pyrazolopyrimidine (or Formycin) analogues, are particularly useful in spectroscopic investigations of the PNP active site, and possibly of other enzymes.

3.2.5 Substrates/Inhibitors of Adenosine Deaminase (ADA). Adenosine deaminase (ADA) is a key enzyme in purine metabolism and plays an important role in maintenance of immune competence. It is specifically involved in a number of pathological states, e.g. overproduction of the enzyme has been associated with hemolytic anemia, and ADA deficiency leads to severe combined immunodeficiency disease (SCID).[131]

The fluorescent 8-azaAdo (Table 2), as well as its 9-deaza congener, Formycin A (FA), are both excellent substrates of ADA, suitable for continuous monitoring of ADA levels in

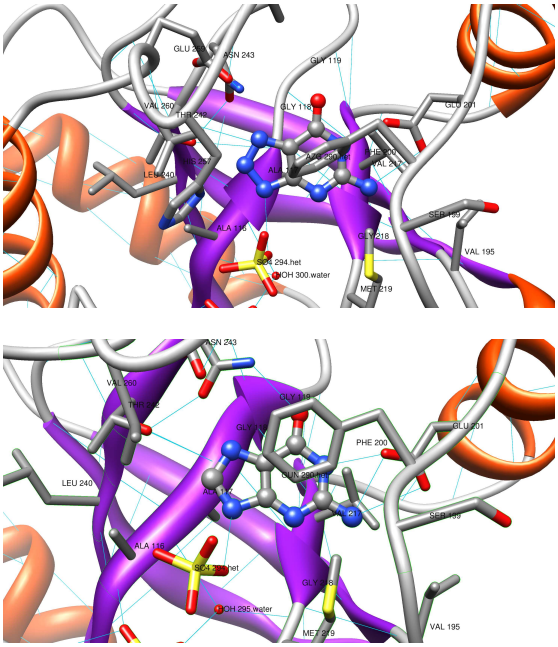

Fig. 6 Binding sites of human PNP with 8-azaGua (upper), and guanine (lower)

cell extracts. The applicability of FA for this purpose was long ago described.[161] It has the marked advantage that, because of its C-C glycosidic linkage, it is resistant to purine nucleoside hydrolases, as well as to PNPs from both mammalian and bacterial sources.

ADA inhibitors are considered as promising anti-inflammatory drugs, and for treatment of leukemia. One such inhibitor, the naturally occurring Pentostatin (the R-epimer of 8-hydroxy-2-deoxycoformycin), has been approved for treatment of hairy cell leukemia.[162] However, because of possible stimulation of adenosine receptors by some ADA inhibitors,[43] interest in development of more selective inhibitors continues unabated.

Many known inhibitors are nucleoside analogues or alkylated adenines, the latter including (+)-erythro-9-(2-hydroxy-3-nonyl)adenine, known as (+)EHNA, a ground-state inhibitor with a K_i in the nM range, and Pentostatin , a transition-state inhibitor, further discussed below. Current efforts to develop potent and specific inhibitors are now based largely on the initially postulated mechanism of the deamination reaction, involving stereospecific addition of water across the 1,6-double bond of adenosine to yield the tetrahedral intermediate (Fig. 7), followed by elimination of ammonia to yield inosine. This proposed mechanism has been confirmed by ^{13}C NMR spectroscopy,[163] X-ray crystallography[164] and Raman spectroscopy.[165]

The foregoing mechanism was subsequently invoked to interpret the inhibition of ADA by purine riboside (nebularine)

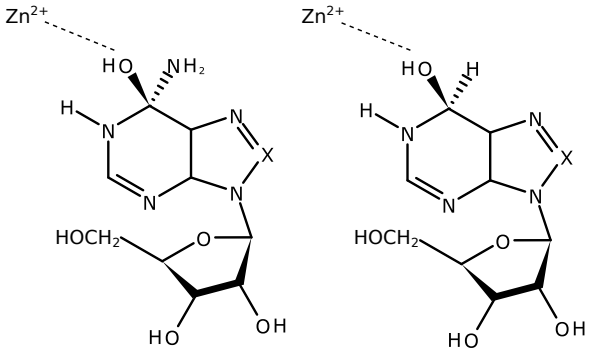

Fig. 7 Structure of the transition state of the ADA-catalyzed deamination of Ado and 8-azaAdo (left) and of hydrated nebularine (X = C) or 8-azanebularine (X=N), bound to the active site (right).

via formation of 6-hydroxy-1,6-dihydronebularine. If correct, then analogues of nebularine with an increased propensity for hydration of the 1,6-bond should exhibit enhanced affinity for the enzyme. And, in fact, whereas nebularine exhibited a K_i of 16 μM, 8-azanebularine, with a K_i of 0.04 μM, was 400-fold more potent.[166]

3.2.6 Adenosine to Inosine RNA editing. The numerous post-transcriptional modifications of mRNA transcripts (pre-mRNA), referred to as RNA editing, include the enzymatic deamination of one or more cytidine and adenosine residues residing at specific sites in the pre-mRNA substrate. The enzymes responsible for deamination of adenosine residues are known as Adenosine Deaminases that act on RNA, ADAR1 and ADAR2, the most widely studied. The resulting generation of inosine, which is decoded as guanosine during translation, leads to introduction of amino acids into a product not encoded by the gene.[167]

Ado→Ino editing is unique in organisms with developed tissue systems (metazoa), and occurs in mRNAs that code for proteins essential to functioning of the nervous system.[168] Some viruses generate RNAs that are targets for editing. Hence selective inhibitors of editing may be potential therapeutic agents.

The structure of the active site of the ADARs suggests that the mechanism of deamination resembles, in some respects, those of adenosine and cytidine deaminases. Both make use of a zinc-bound water molecule to carry out hydrolytic deamination. This is consistent with the finding that mutation of the active site residues of ADARs involved in zinc binding leads to loss of activity. It is, furthermore, quite striking that ADAR enzymes share little sequence homology with adenosine deaminases (ADA), but have conserved sequences similar to the consensus sequence of the active site of cytidine deaminases (CDA), hence appear to have evolved from the CDA family.[169] They do not deaminate free cytidine residues.

There are some marked differences in base recognition strategies between ADA and ADARs. For example, whereas 2,6-diaminopurine riboside is a good substrate of ADA, it is not a substrate for an ADAR. And 7-deazaAdo, which is not a substrate or inhibitor of ADA, is a good substrate for ADAR2, but not ADAR1. Coformycin, a potent inhibitor of ADA, does not inhibit ADARs.[170]

Studies on the reactivity of adenosine analogues incorporated at editing sites have proven quite informative. In particular, replacement of a susceptible adenosine residue in an RNA substrate by 8-azaAdo led to a large increase in the rate of deamination, in accord with both experimental and theoretical predictions that 8-aza substitution facilitates hydration across the N(1)-C(6) double bond.[1,171,172] Furthermore, whereas incorporation of 8-azanebularine into an editing site of an RNA substrate recognized by human ADAR2 resulted in high-affinity binding (K_D = 2 nM), similar incorporation of the C(6)-methyl congener of 8-azanebularine, which is known to inhibit covalent hydration, leads to a dramatic decrease in binding affinity, with K_D = 150 nM.[173]

ADAR enzymes also differ from adenosine deaminases in that the adenosine to be deaminated must be located in a duplex structure of at least 15-20 base pairs for deamination to occur. One consequence of this is a requirement for a conformational change of the duplex substrate so that the base to be edited can be flipped out of the helix to access the active site of the enzyme. Support for this has been indirectly demonstrated by preparation of RNA duplex substrates incorporating the fluorescent 2-aminopurine riboside at various positions, including a known editing site. The emission of 2-aminopurine riboside was appreciably (but not fully) quenched on incorporation, as a result of base stacking. Subsequent addition of ADAR resulted in a 3-fold enhancement of emission of only the 2-aminopurine riboside incorporated at an editing site, testifying to its being flipped out of the duplex. No such effect was observed with 2-aminopurine riboside incorporated into single-stranded RNA.[174]

The foregoing may prove to be more instructive by incorporation at an editing site of a fluorescent purine riboside analogue which base pairs with the uridine in the complementary strand (so that its emission would probably be more effectively quenched), and is susceptible to covalent hydration. One such promising candidate is 8-azaAdo (cf. Veliz et al.[175]). It exhibits good emission, with λ_{max} 352 nm (see Table 3). And, when located at an editing site, it is a far better substrate for an ADAR than Ado (see above). Another potential candidate is the strongly fluorescent Formycin A (8-aza-9-deazaAdo), an excellent substrate for free adenosine deaminase (see above).

No effective low-molecular weight inhibitors of ADARs are presently known. Veliz et al.[175] reported that 8-azanebularine, but not the parent nebularine, as the free nucleoside, very weakly inibits ADAR 2, with an IC_{50} of 50 mM, It would have been interesting to examine the effect of 8-aza-

nebularine on ADAR1, as well as trials with other 8-azapurine nucleosides, bearing in mind that RNA editing may have a profound impact on drug discovery and development, and lead to major discrepancies between the results of pharmacological studiess in vtro and in vivo.[176]

3.2.7 Guanine deaminase (Guanase, Cypin). This enzyme, which catalyzes the hydrolytic deamination of Gua to Xan, effectively eliminates Gua from further utilization as a Gua nucleotide. The enzyme is not ubiquitous, is generally absent in lymphoid tissues, and is variously expressed in human liver, brain and kidney. Abnormally high levels exist in patients with liver diseases, including those resulting from infection with hepatitis B and C viruses. Consequently, serum enzyme levels are a highly sensitive indicator of hepatitis and hepatoma. Elevated levels of the enzyme are also found in the cerebrospinal fluids of patients with multiple sclerosis.[177]

An important function has been ascribed to Guanase in the brain. Dendrite morphology is crucial for normal neuronal communication, including spatial and functional assembly of signal transduction pathways at synaptic sites and patterning of dendrites and their branches, and regulates how a postsynaptic neuron receives information from presynaptic neurons. Following isolation of a protein named Cypin (postsynaptic density, PSD-95, protein), which decreases localization of membrane-associated guanylate kinases that play a role in maturation of excitatory synapses, it was found that neurons overexpressing Cypin display increased dendritic branching. Furthermore, Cypin was found to be a guanine deaminase, making it a promising candidate for identifying mechanisms by which purines are known to regulate neuronal morphology.[178]

Substrates, at pH 6, include the 1-methyl-, but not the 3-, 7- and 9-methyl-, guanines.[179] At the same pH, 8-azaGua is a very good substrate. Although its K_m is about 2 orders of magnitude higher than that for Gua, its V_{max} is 10-fold higher, and it has been widely used as a substrate for spectrophotometric assays of the enzyme.[180] Bearing in mind that the pK_a of 8-azaGua is 6.5 (Table 1), it exists at physiological pH, unlike guanine, as a mixture of the neutral and monoanionic species. Lewis and Glantz[181] reported that the optimum pH was 6.8 with Gua as substrate, whereas a sharp optimum was observed at pH 6 with 8-azaGua. This suggests that the neutral form of the latter is the substrate.

Development of a more sensitive assay for levels of Guanase in various pathological states would be highly desirable. However, continuous monitoring of deamination of the fluorescent 8-azaGua by emission spectroscopy is precluded by the fact that the high emission of the product, 8-azaXan, at 420 nm (ϕ = 0.23 at pH 3.5) overlaps that of the substrate with maximum emission at 390 nm (see Table 1). But application of HPLC or capillary electrophoresis, with fluorimetric monitoring of the product, would clearly lead to a sensitivity at least two orders of magnitude higher than existing methods.

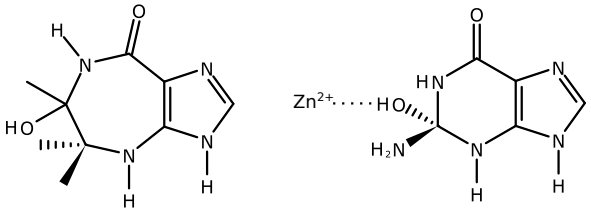

Fig. 8 Azepinomycin (left), a putative transition-state analogue of Guanase, and the proposed transition state structure.[182,183]

There are numerous reports on development of Guanase inhibitors (see citations in Ujjinamatada et al.[182]), but none with a K_i in the submicromolar range. Furthermore, some reported inhibitors lack selectivity, in that they also inhibit other enzymes such as adenosine deaminase and xanthine oxidase.

A relatively recent, and rational, approach[182] aimed at the design of more potent inhibitors, was based on the known reaction mechanism, involving an active site Zn^{2+}, which forms a tetrahedral complex with His53, Cys83, Cys86 and a water molecule. Glutamate55 then abstracts a proton from the Zn^{2+}-activated water molecule to form a hydroxide nucleophile, at the same time enabling protonation of the N(3) of Gua, leading to the transition-state intermediate shown in Fig. 8, supported by analysis of the crystal structure of the Bacillus subtilis enzyme.[183]

In accordance with the foregoing, the naturally occurring azepinomycin (Fig. 8), a ring-expanded purine analogue, considered a mimic of the transition-state of the enzyme-catalyzed deamination reaction, is an inhibitor, but surprisingly only a moderate one, with $IC_{50} \sim 5\ \mu$M. Introduction of an additional potential Zn^{2+}-coordination site on azepinomycin, as shown in Fig. 8, did not, as anticipated, enhance inhibitory activity.[182]

Alternatively, bearing in mind that 8-azaGua is a very good substrate, a more promising inhibitor might be the corresponding hitherto unknown 2-aza-azepinomycin (Fig. 8).

3.3 Purine salvage pathway: Chemotherapy of malaria with 8-azapurines

Like all protozoan parasites, *Plasmodium falciparum*, the most lethal responsible for malaria, is unable to synthesize the purine ring *de novo*, and is strictly dependent on the salvage of purines from the host cell (salvage pathway), which are then converted to purine nucleotides for DNA and RNA synthesis (for review, see el Khouni[112]). An essential enzyme in this pathway is the parasite hypoxanthine/guanine/xanthine phosphoribosyl transferase (HGXPRTase, 6-oxopurine HGXPRTase), which catalyzes formation of 6-oxopurine nucleo-

side 5'-monophosphates from the parent base and 5-phospho-α-D-ribose-1-pyrophosphate.

A recent promising approach to chemotherapy of malaria,[95] profiting from the purine salvage pathway, was based on a search for purine base analogues which are good substrates for the *P. falciparum* HGXPRTase, but not for the host (mammalian) HGPRTase (for which Xan is not a substrate). Such analogues, once inside the cell, may then be converted by the parasite enzyme to the corresponding toxic nucleotides, similar to the mechanism of the clinically approved antitumour drug 6-mercaptopurine.[184]

Screening a series of purine analogues revealed that three of them, 6-chloroguanine, 8-azaHx and 8-azaGua, potently discriminated between the parasite and human enzymes, the more effective being 8-azaHx, for which the k_{cat}/K_m was almost 350-fold higher than that for the human enzyme. For 8-azaGua, it was 80-fold higher. By contrast, 8-azaXan was neither a substrate, nor a competitive inhibitor, although Xan is a weak substrate of the parasite enzyme.

Both 8-azaHx and 8-azaGua inhibited the growth of *P. falciparum* cells in erythrocytes *in vitro* at micromolar concentrations, 8-azaGua being 2-fold more effective than 8-azaHx, notwithstanding that 8-azaGua is a less effective substrate of the enzyme in vitro than 8-azaHx (see above). This difference may conceivably be due to differences in ability of the two purine analogues to traverse the parasite membrane, e.g. both analogues, but not 8-azaXan, inhibited the uptake of [^{3}H]hypoxanthine, but 8-azaGua was 15-fold more effective than 8-azaHx. In this context, it is of undoubted interest that, for *P. falciparum*, uptake of purines is strictly under the control of the plasma membrane permease PfNT1,[185] and this permease is itself a potential therapeutic target for malaria. It should, however, be recalled that 8-azaGua has been reported to be toxic to human cells, as a result of its incorporation into RNA (see Section 3.1).

In attempts to account for the discrimination between the human and parasite PRTases, the authors erroneously assumed that all the base analogues, including Xan, 8-azaHx and 8-azaGua, are uncharged molecules, existing only as equilibrium mixtures of the N(7)-H and N(9)-H prototropic tautomers. This is obviously not applicable to the 8-aza analogues (see Gasik et al.[186]) which are exclusively or predominantly monoanions at the pH employed, 7.4 and 8.5 (see Section 2) notwithstanding that both were more efficient substrates for the parasite enzyme at pH 7.4 than at pH 8.5.

3.4 8-azapurine derivatives as inhibitors of protein kinases

Reversible phosphorylation of proteins by protein kinases, of which more than 500 are encoded in the human genome alone, is the predominant mechanism whereby signal transduction

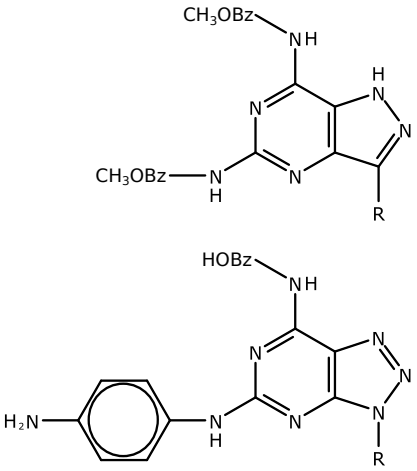

Fig. 9 Pyrazolo[4,3-d]pyrimidine analog of myoseverine (upper, R = isopropyl), active as an inhibitor of CDK and tubulin polymerization, and one of the synthesized 8-azaDaPur analogues(lower), the most active as a p53 inducer.

pathways operate by transfer of information between specific cellular sites. Dysfunctions of such kinases have been implicated in a variety of pathological states, ranging from cancer, through Altzheimers disease and diabetes. Hence considerable efforts are directed to development of selective inhibitors of these enzymes, as well as their potential as chemotherapeutic agents.

3.4.1 Inhibitors of cyclin-dependent kinases. Inhibitors of cyclin-dependent kinases are among the most promising anti-cancer agents, selectively inducing apoptosis of transformed cells[187] Derivatives of 2,6-diaminopurine, substituted at the 2, 6 and 9 positions, are amongst the most potent inhibitors of these kinases, particularly CDK1, CDK2 and CDK5, with IC50 values in the nanomolar range.[188]

Several analogous 8-azapurine and pyrazolo[4,3-d]pyrimidine derivatives were synthesized and examined as potential alternatives. Although their in vitro IC_{50} values towards the CDK1 and/or CDK2 kinases were only in the low micromolar range, some of them, especially the 8-azapurine derivatives, exhibited promising behaviour in biological tests, showing inhibition of proliferation of several neoplastic cell lines. This activity may be related to the observed induction of wild-type tumor suppressor p53 in these lines.[189]

It is of some interest that the pyrazolo[4,3-d]pyrimidine analogue of myoseverin (2,6-di-p-methoxybenzyl derivative of DAP), a strong tubulin polymerization inhibitor (Fig. 9), is also an inhibitor of CDK1, CDK2 and CDK7.[190].

In contrast to the 2,6-diaminopurine derivatives, which are only weakly fluorescent, the N2, N6- disubstituted 8-azapurine analogues exhibit fairly intense fluorescence in neutral aqueous medium, centered at ~370 nm (see Section 2).

Weaker, but readily detectable, emission is observed for the corresponding pyrazolopyrimidine analogues. This points to the possibility of studying CDK-ligand interactions by spectroscopic methods.

3.5 8-Azapurine fluorescent probes of mechanisms of catalytic RNAs (ribozymes)

Charged amino acid residues play key roles in catalysis by protein enzymes, e.g. lysine and arginine function as oxyanion holes to stabilize charge development in the transition state, while transfer of protons is mediated by cycling of histidine residues between the neutral and positively charged forms. Natural ribozymes utilize many of the strategies of protein enzymes, such as the the role of metal ions, and general acid-base and electrostatic catalysis. The role of charged nucleobases in catalytic RNAs (ribozymes) has been concisely reviewed by Bevilacqua and coworkers.[191]

The mechanism of enzymatic activity of catalytic RNAs involves metal-ion and/or nucleobase catalysis (for reviews, see Fedor,[192,193] Lonnberg and Lonnberg,[194] Bevilaqua and Yajima,[195] and Wilcox et al.[191]). In general, ribozymes are divided into two classes with respect to their catalytic strategy. Large ribozymes, like RNase P, and group I and group II self-splicing introns, usually require divalent metal ions for their activity. Small ribozymes, exemplified by the hairpin or hammerhead ribozymes, are fully active in the absence of these ions, pointing to direct involvement of active nucleotides in the catalytic process. In consequence, efforts are directed to identify catalytically active nucleotides and to elucidate the role of the ionic forms of their bases. Isosteric purine analogues, such as 8-azapurines, particularly fluorescent ones, have proven to be exceptionally useful probes for this purpose.[8,16,17]

One way in which 8-azapurines may contribute to mechanistic studies of ribozyme activity is by following the proton transfer reaction of the N(1)-H in Gua or to N(1) in Ade residues, presumed to be involved, inter alia, in RNA cleavage catalyzed by the hairpin ribozyme.[195,196] The intrinsic pK_a values are measured, and compared with pH-activity profiles. Typical approaches to this problem are via NMR[197] crystallography,[196,198] or kinetic isotope effects.[199] However, thanks to the relatively high fluorescence of 8-azaAdo and 8-azaGuo (the latter in its monoanionic form), this can be performed fluorimetrically, by titration of the modified ribozyme in which a presumed critical Gua residue is replaced by 8-azaGua,[8,13,17] or, alternatively, a suspected catalytically active Ade is replaced by 8-azaAde.[16]

Results reported by Fedor et al.[13,16,17] suggest that the intrinsic pK_a of 8-azaGua in the hairpin and glmS ribozymes is elevated (ca. >9 and 8.9, respectively), so that in both cases the 8-azaGua residue remain protonated at pH 7. Simultaneously, the pH-activity profile pointed to a pK_a of 7.9 for the glmS, and $\sim$6.5 for the hairpin rybozyme, respectively, thus quite distant from the former values, suggesting that, in both instances, Gua residues participate in catalysis as the neutral species. When, in the hairpin ribozyme, the active Ade was replaced by 8-azaAde, the intrinsic pK_a determined fluorimetrically correlated well with the pK_a of the pH- activity profile, indicating that the neutral non-protonated 8-azaAde residue is involved in catalysis.[16] The authors proposed general acid-base catalysis involving both Gua (at position 8, acid) and Ade (at position 38, base) in the transition state.

Some ribozymes (e.g. group I intron-splicing ribozymes) require a free nucleotide (GMP, GTP) or a nucleoside, like Guo, as co-substrate.[200–204] Binding of Guo was found to be a rate-limiting step for the *Tetrahymena* ribozyme at neutral pH.[201,205] And 8-azaGuo was found to be one of the best non-natural co-substrates for this reaction.[200] The apparent K_m for 8-azaGuo ($\sim$25 μM) compares well with that for Guo ($\sim$30 μM), and the k_{cat}/K_m is more than 3-fold higher ($\sim 3\times10^4$ $M^{-1}min^{-1}$). Hence 8-azaGuo and, possibly, some reated derivatives, are likely suitable tools for mechanistic and kinetic studies of this class of reactions *via* a fluorescence approach.

3.6 Adenosine receptors

Adenosine receptors, coupled with various G proteins, regulate the activities of intracellular factors such as cAMP. cGMP, phospholipases A or C, etc, in mammalian cells.[206] They consist of 4 sub-types, and there is considerable interest in development of agonists/antagonists selective for each subtype. Hundreds of more or less selective ligands, largely adenine and xanthine analogues, are now known.[188,207,208] Selective ligands of the A1 receptor are considered pharmacologically useful in hypertension, and kidney and cardiac therapies, while anti-parkinsonian activities have been reported for A2-type antagonists (Legraverend and Grierson,[188] and ref. cited).

Giorgi and Scartoni[43] have recently reviewed various applications of 8-azapurine derivatives in this field, with emphasis on receptor binding studies. They did not, however, take into account the fluorescence emission properties of various 8-azaAde and 8-azaXan derivatives characterized as receptor ligands (agonists and antagonists). This aspect clearly requires more attention, bearing in mind that fluorescence spectroscopy has already been successfully applied to studies on receptor dynamics.[209–213]

4 Summary and Perspectives

We have, in the foregoing, presented the most comprehensive, to date, review of the physico-chemical properties of

the 8-azapurines and their nucleosides and nucleotides, with particular emphasis on the intrinsic fluorescence of some of them. Since all of these are isosteres (isomorphs) of the natural purines, it is to be expected that they may simulate, or compete with, the latter in various biochemical pathways involving natural purines and their glycosides. Several examples are presented, e.g. the ability of some azapurines to replace catalytically active purines in ribozymes, and in RNA editing. The fluorescence properties of some 8-azapurines have been profited from to accurately delineate the mode of action of purine nucleoside phosphorylases and phosphoribosyltransferases, and to monitor their activities by fluorescence spectroscopy. Overall, it is clear that the 8-aza purines, and their fluorescence emission properties, are useful tools in future studies on metabolic pathways involving purines and purine analogues.

Acknowledgements

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