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Fluorescence emission properties of 8-aza analogues of caffeine, theophylline, and related *N*-alkyl xanthines ☆

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ABSTRACT

Fluorescence emission properties of 8-azacaffeine, 8-azatheophylline and other N-alkylated 8-azaxanthines (8-azaXan) have been examined. It is shown that N-methylated 8-azaxanthines, as well as 8-azatheophylline, are highly fluorescent in aqueous medium as the neutral, and, in some instances, also as the monoanionic, forms. 8-Azacaffeine exhibits moderate emission, but its isomer, 1,3,8-trimethyl-8-azaXan, is highly fluorescent. All three 8-azaxanthines monomethylated on the triazole ring, as well as 8-azaxanthosine, exhibit increased acidity in the excited state. Some fluorescent pyrazolo[4,3-*d*]pyrimidine-5,7-diones, xanthine congeners of pyrazolo[4,3-*d*]pyrimidines, are also reported. Many of these are good fluorescent probes in enzymatic, receptor binding, and nucleic acid systems, some examples of which are presented. In particular, 8-azaXan is an excellent fluorescent probe for purine nucleoside phosphorylases, as a fluorogenic substrate in the reverse, synthetic pathway.

1. Introduction

Fluorescent nucleic acid base analogues, such as pyrazolopyrimidines, 8-azapurines (triazolo[4,5-*d*]pyrimidines), and the corresponding nucleosides and nucleotides are useful as fluorescent reporter molecules in biological systems, along with a variety of recently introduced dye-tagged nucleosides, nucleotides and oligonucleotides.[1–5] In particular, the fluorescent isosteric analogue of adenosine, formycin A (8-aza-9-deazaadenosine), has been successfully applied to study protein–ligand interactions in a number of enzyme systems,[6–9] and fluorescent purine alkoxyphosphonates, including those of the 8-azapurine series, have proven useful as fluorescent probes of purine-nucleoside phosphorylases.[10,11] Other examples include studies of the ionic states of catalytic nucleic acids, such as ribozymes, by monitoring the pH-dependent emission of 8-azaGuo residues substituted for Guo,[12] and many applications of 2-aminopurine fluorescence to the study of nucleic acid interactions and dynamics.[13–15]

In the course of our studies on the fluorescence of 8-azapurines[16,17] we discovered strong room-temperature emission of 8-azaxanthine (8-azaXan) and its 8-methyl derivative in aqueous media.[18] *N*-methyl derivatives of 8-azaxanthine were long ago characterized,[19–21] but their room-temperature fluorescence has, to our knowledge, been overlooked in the literature. We here present the fluorescence emission properties of other N-methylated 8-azaxanthines, including 8-azacaffeine, its biologically active[22] isomer 1,3,8-trimethyl-8-azaXan, and 8-azatheophylline, as well as some xanthine analogues of pyrazolo[4,3-*d*]pyrimidines (8-aza-9-deazapurine, see Scheme 1).

Detailed analysis of spectral features of various substituted xanthines was found useful in our studies of purine nucleoside phosphorylase (PNP) from various sources. We have already shown[23] that 8-azaxanthine is a moderately good substrate for the *Escherichia coli* PNP-II in the reverse, synthetic pathway. Moreover, this reaction gives at least two products, that is, 8-azaxanthosine, and, additionally, a non-typical, highly fluorescent nucleoside (λ_{max} = 440 nm at pH 2–11), tentatively identified as the 8-β-D-riboside.[23] Furthermore, the recombinant calf spleen PNP in the same type of reaction gives another product which is clearly distinct from the 9-β-D-riboside (unpublished). Identification of these products, and factors affecting the reaction outcome, is important for further applications of PNP in nucleoside synthesis, reviewed recently by Mikhailopulo.[24]

Additionally, spectral studies of macromolecular complexes with fluorescent ligands undergoing ground- and/or excited-state

Abbreviations: Xan, xanthine; Xao, xanthosine; 8-AzaXan, 8-azaxanthine; 8-AzaXao, 8-azaxanthosine; ϕ, fluorescence quantum yield; PNP, purine-nucleoside phosphorylase.

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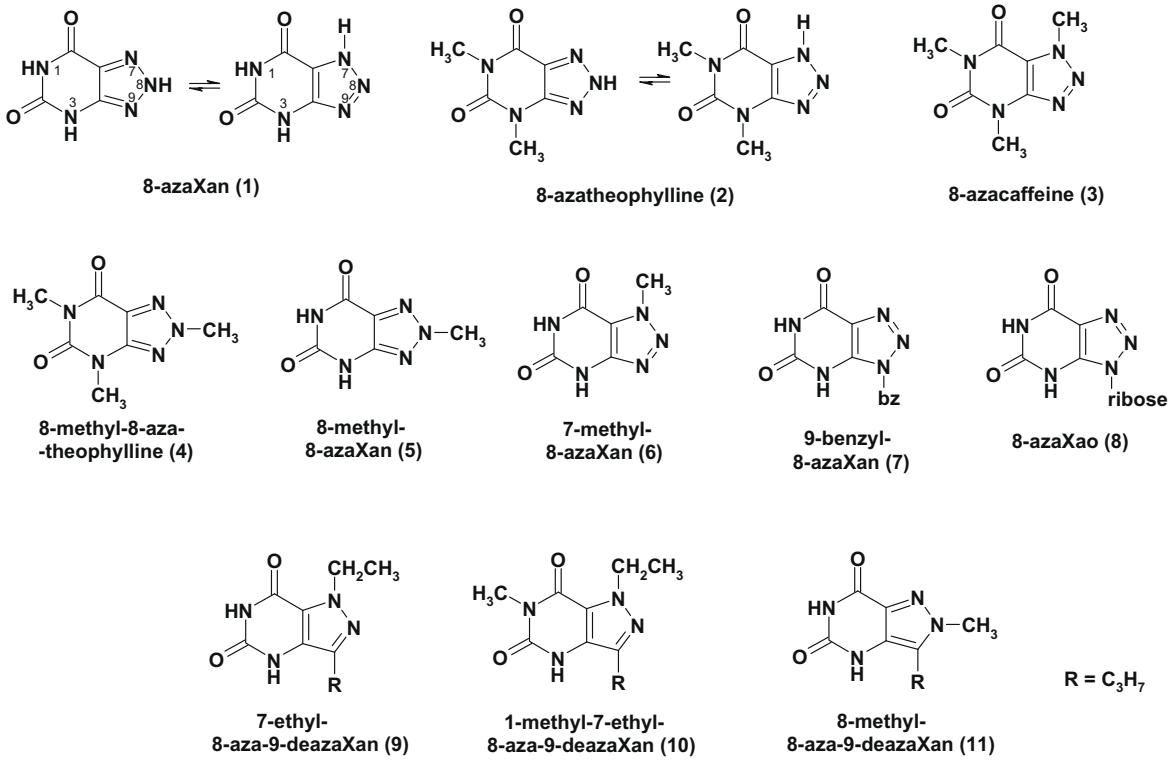


Scheme 1. Fluorescent analogues of xanthine, examined in this paper. Note that purine numbering, instead of UPAC numbering, is maintained throughout. The neutral forms of 8-azaXan (**1**) and 8-azatheophylline (**2**) are shown in their two, experimentally detectable, protomeric forms.

tautomerism has been shown to provide information on hydrogen bonding patterns within the binding site,[25,26] and its possible changes. For example, 6-methylformycin A, complexed with the *E. coli* PNP-I, adopts the imino structure in the absence of phosphate, but is converted to the amino form in the ternary complex enzyme–nucleoside-phosphate.[7] Tautomerism of the purine substrate in the binding site of the enzyme appears to be an important factor affecting the site of its enzymatic ribosylation.

Additional interest in the foregoing stems from the fact that many *N*-alkyl derivatives of 8-azaxanthine are potent antagonists of adenosine receptors,[22] and 8-azaXMP is known as a potent inhibitor of the yeast orotidine-5′-phosphate decarboxylase.[28] The parent 8-AzaXan is a good selective inhibitor of urate oxidase (uricase).[27] Within a program designed to infer the in vivo efficacy, specificity and toxicity of chemical leads, NMR metabolic profiling has recently been employed to demonstrate that 8-azaXan inhibits hyphal growth of *Aspergillus nidulans,* and that this occurs via specific in vivo inactivation of uricase.[29] Neutron diffraction is presently being applied to crystalline complexes of urate oxidase with 8-azaXan to determine the protonation state of the latter within the active site.[30]

The investigated xanthine and xanthosine analogues are consequently promising candidates as fluorescent probes in these, and other enzymatic systems, including those involved in xanthine methylation during caffeine biosynthesis, recently reviewed by Kulikowska et al.[31]

2. Results and discussion

2.1. Fluorescence of 8-azatheophylline and 3-methyl-8-azaxanthine: comparison with 8-azaxanthine

One of the oldest known purine analogues, 8-azatheophylline (1,3-dimethyl-8-azaXan, **2**),[32] is a highly fluorescent compound, with $\lambda_{max} \sim 353$ nm, and fluorescence quantum yield (ϕ) of 0.18 at pH ~ 3 (Table 1, Fig. 1), but non-fluorescent at neutral pH, where it exists as a monoanion, due to dissociation of the triazole proton ($pK_a \sim 4.6$). The relatively strong acidity of the triazole proton is typical for 8-azapurines,[19] including 8-azaXan (**1**, $pK_a \sim 4.8$[18]). Similar emission ($\lambda_{max} \sim 355$ nm) is exhibited by 3-methyl-8-azaxanthine (Table 1). Both compounds emit only as the neutral species, that is, at pH < 5 (Table 1), like the parent 8-azaXan.[18] However, the emission spectra of both are blue-shifted by ~70 nm relative to that of 8-azaXan, as shown in Figure 1 and Table 1. This is in accord with the previously postulated phototautomeric behavior of 8-azaXan, where the long-wavelength emission band is due to formation of the rare anionic species, with N(3)H dissociated in the excited state (Scheme 2).[18]

In highly fluorescent aromatic compounds, the fluorescence excitation spectrum usually overlaps the UV-absorption spectrum.[33] This does not apply to 8-azaXan, nor to 8-azatheophylline, where excitation spectra are red-shifted relative to absorption by ~8 nm in aqueous medium (see Fig. 1). A similar effect was observed in anhydrous alcoholic media (not shown). We interpret this phenomenon as resulting from ground-state annular N(7)H–N(8)H tautomerism in 8-azaXan and 8-azatheophylline. The latter, long ago characterized by l'Abbe et al.[34] by means of ^{15}N NMR spectroscopy, demonstrated that in DMSO it exists in the neutral form as a mixture of protomers with 20% as the N(8)H, 80% as the N(7)H, and no detectable traces of the N(9)H. These findings are supported by the spectral properties of 8-azatheophylline derivatives monomethylated on the triazole ring, described below.

2.2. Fluorescence of trimethyl 8-azaxanthines

8-Azacaffeine (1,3,7-trimethyl-8-azaXan, **3**), is only weakly fluorescent in aqueous medium, with $\lambda_{max} \sim 350$ nm, and quantum yield, $\phi \approx 0.015$ (Table 1). Its fluorescence excitation spectrum is

Table 1
Spectral parameters and dissociation constants (pK_a) of 8-azaxanthine, its *N*-methyl derivatives, and some 8-aza-9-deazaxanthines in aqueous medium

Compound	pK_a	Form	Solvent, pH	Absorbance:		Fluorescence		
				λ_{max} [nm]	ε_{max} [M^{-1} cm^{-1}]	λ_{max} [nm]	ϕ	τ [ns]
8-AzaXan (**1**)	4.8; 9.8	Neutral[a]	5 mM acetic acid, pH 3.5	263	6500	420	0.22	9.0
		Anion	Phosphate, pH 7	265	8500	-	<0.005	-
8-Methyl- (**5**)	7.6 (7.3[b])	Neutral[a]	Phosphate, pH 6	272	8700	420	0.5	12
		Anion	10 mM carbonate, pH 10	299	6450	420	0.60	12
7-Methyl- (**6**)	7.3 (7.0[b])	Neutral[a]	5 mM acetic acid, pH 3.5	273	6030	400	0.13	4.3
		Neutral	Methanol	275	nd	350, 430	<0.005	nd
		Anion	10 mM carbonate, pH 10	303	5600	400	0.23	4.9
9-Benzyl- (**7**)	5.4	Anion	10 mM phosphate, pH 8	278	7600	365	0.11	nd
		Neutral	5 mM acetic acid, pH 3.5	255	9100	365, 430	~0.1	nd
3-Methyl-	4.5; 11.4	Neutral	5 mM acetic acid, pH 3.5	268	5400	355	~ 0.2	nd
1,3-Dimethyl- (**2**)	4.6 (4.5[b])	Neutral	5 mM acetic acid, pH 3.5	271	10,000	353	0.18	3.65
		Anion	0.001 N KOH	266	13,000	-	<0.005	-
1,3,7-Trimethyl- (**3**)	-	Neutral	H_2O	280	7800	350	0.013	nd
1,3,8-Trimethyl- (**4**)	-	Neutral	H_2O	276	10,000	355	0.44	3.48
8-AzaXao (**8**)	4.65, 4.7[b]	Anion	5 mM phosphate, pH 7	277	8350	363	0.10	nd
		Neutral	1 mM HCl	255	8750	365, 430	~0.1	nd
8-Aza-9-deaza-Xan 9-propyl-7-ethyl- (**9**)	9.24	Neutral	5 mM phosphate, pH 7	297	nd	375	0.025	nd
		Anion	0.001 M KOH	317	nd	367	0.15	nd
9-Propyl-7-ethyl-1-methyl- (**10**)	9.5	Neutral	5 mM phosphate, pH 7	296	6950	365	0.02	nd
		Anion	0.001 M KOH	323	7850	397	0.04	nd
9-Methyl-8-methyl- (**11**)	9.4	Neutral	5 mM phosphate, pH 7	294	nd	370	0.029	nd
		Anion	0.001 M KOH	316	nd	375	0.19	nd

Fluorescence quantum yields (ϕ) were determined relative to tryptophan (0.15), with excitation at 280 nm. Data for 8-azaXan and 8-methyl-8-azaXan were reported earlier.[18]

[a] Refers to ground state; on excitation, rapid proton dissociation occurs.

[b] Fluorimetric titration.

slightly red-shifted relative to its UV absorption spectrum (Fig. 2), possibly due to the so-called cage effect.[33] By contrast, the isomeric 1,3,8-trimethyl-8-azaXan (**4**) is highly fluorescent, with a quantum yield reaching 0.44 (Table 1), and a fluorescence excitation spectrum quite similar, but not identical, to the UV absorption spectrum (Fig. 2). We were unable to isolate the pure 9-methyl derivative of 8-azatheophylline, due to its instability,[21] but preliminary inspection of TLC eluates (λ_{max} = 259 nm[20]) indicate this compound to be virtually nonfluorescent in aqueous medium. We therefore conclude that the observed strong emission of 8-azatheophylline, 8-azaXan and 3-methyl-8-azaXan must be due to the N(8)H tautomer, since the only strongly fluorescent *N*-alkyl 8-azatheophylline derivative is that alkylated on N(8). This conclusion is additionally supported by the near identity of fluorescence decay times of 8-azatheophylline (**2**) and 1,3,8-trimethyl-8azaXan (**4**, Table 1).

2.3. Emission properties of 8-azaxanthines methylated on the triazole ring: increased excited-state acidity of N(3)H

In apparent contrast to the foregoing, all three 8-azaXan derivatives, monomethylated on the triazole ring, emit strongly in the range of pH 2–11, with emission maxima red-shifted relative to that of 8-azatheophylline (355 nm), but similar to that observed in 8-azaXan (420 nm). It was earlier demonstrated that the strong emission of 8-azaXan (**1**) and 8-methyl-8-azaXan (**5**) in aqueous

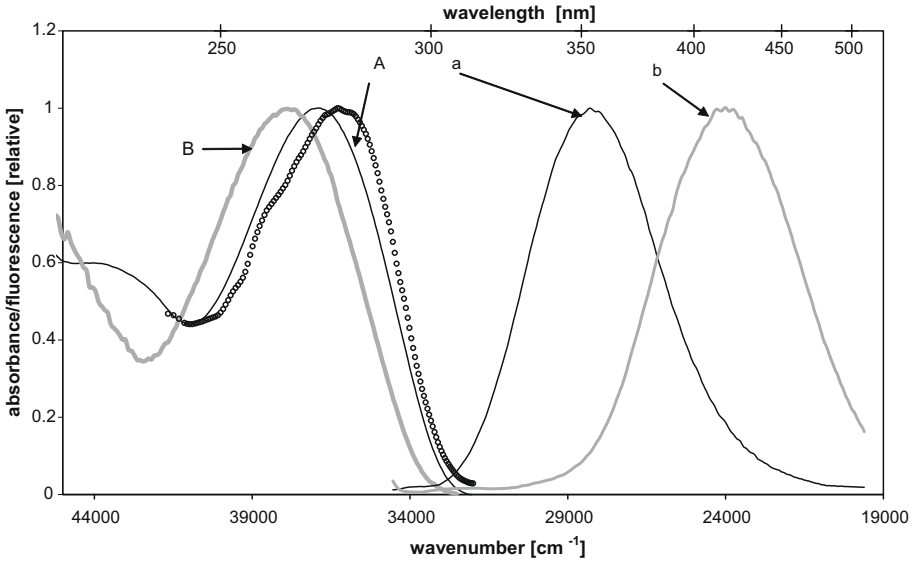
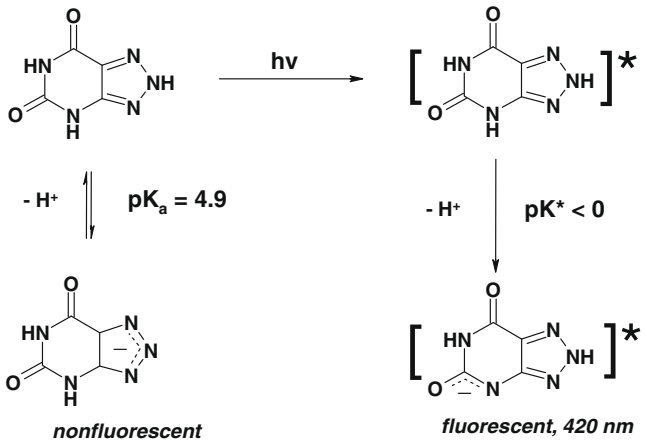


Figure 1. UV absorption (A), fluorescence emission (a), and fluorescence excitation (○ ○ ○) spectra of 8-azatheophylline (**2**); and, for comparison, the previously reported[7] UV absorption (B), and fluorescence emission; (b) spectra of 8-azaXan (**1**), all in 10 mM acetate, pH 3.5. All spectra are normalized to unity.



Scheme 2. Origin of the long-wavelength (λ_{max} = 420 nm) fluorescence of 8-azaXan in weakly acidic aqueous medium. Note that this emission is not observed in N(3)-alkylated 8-azaxanthines.

medium is due to the anions resulting from dissociation of the N(3)H which leads to fluorescent anionic species.[18] This anionic fluorescence is also visible in the weakly acidic pH range, due to the excited-state dissociation of the N(3)H, observed directly in 8-methyl-8-azaXan,[18] and postulated for 8-azaXan. The same process occurs in the 7-methyl and 9-benzyl-derivatives of 8-azaXan, as shown below.

The emission maxima of both 8-methyl- (**5**) and 7-methyl-8-azaXan (**6**), 420 nm and 400 nm, respectively, are virtually pH-independent, but the corresponding excitation spectra reveal marked pH-dependence and roughly coincide with the UV absorption spectra both at acidic and basic pH (see Fig 3). It follows that two different ground-state forms, that is, neutral and monoanionic, lead to a single emitting species, at least in aqueous medium, supported additionally by identity of the fluorescence decay times at pH 3 and 9 (Table 1). The emitting species is clearly the monoanion, with N(3)H dissociated, for the following reasons: (a) the Stokes shift at pH > 8 is ~8000 cm^{-1}, while at acidic pH it increases to ~15,000 cm^{-1}, pointing to an excited-state reaction and (b) the observed emission is strongly solvent-dependent,[18] and for 7-methyl-8-azaXan virtually disappears in anhydrous alcohols (Table 1), but can be restored by addition of ~0.4 mM triethylamine (not shown).

9-Benzyl-8-azaXan (**7**) exhibits intense emission at pH 7–9, that is, as the monoanion (pK_a = 5.4), centered at ~370 nm (Table 1). This emission prevails also at pH < 5, due to the postulated N(3)H photodissociation, but is accompanied by an additional long-wavelength band, centered at ~430 nm, which exhibits an almost identical excitation spectrum, virtually in line with UV absorption (data not shown). The calculated Stokes' shift for the long-wavelength band is ca. 16,000 cm^{-1}, pointing to an additional excited-state process, which we tentatively ascribe to keto–enol phototautomerism (under further investigation).

The proposed N(3)H photodissociation can be verified by calculation of the excited-state acidity constant, or pK^*, of these compounds, using Foerster's formula[33]

$$pK^* - pK = 2.1 \cdot 10^{-3}[\nu_{00}(A^-) - \nu_{00}(AH)] \quad (1)$$

where $\nu_{00}(A^-)$ and $\nu_{00}(AH)$ are the wave numbers (in cm^{-1}) of the 0–0 transitions of the deprotonated and protonated forms, respectively. The approximate energies of the 0–0 transitions can be estimated using absorption–emission symmetry, as a crossing point between normalized emission and the lowest-energy absorption band,[35] assuming that the same species is responsible for absorption and emission. In the present case, determination of the 'true' emission spectrum of the neutral form of 7-methyl-8-azaXan (**6**) and 8-azaxanthosine (**8**), is possible only in non-aqueous medium, for example, methanol or isopropanol, since in aqueous medium the photon-induced N(3)H dissociation is nearly complete[18] and emission of the neutral form is not observed (cf. Fig. 3 and Ref. 18).

The estimated pK^* values are listed in Table 2 and show very strong excited-state acidity of all three *N*-triazole-monomethylated 8-azaXan derivatives, as well as 8-azaXao (**8**), which must be ascribed to the significantly decreased electron density on the N(3) nitrogen in the S_1 state, relative to the ground state. By contrast, the excited-state acidity of 8-azatheophylline (**2**) is somewhat reduced relative to that of the ground-state, indicating in this case some increase of electronegativity of the triazole nitrogens upon electronic excitation. Although pK^* values calculated according to

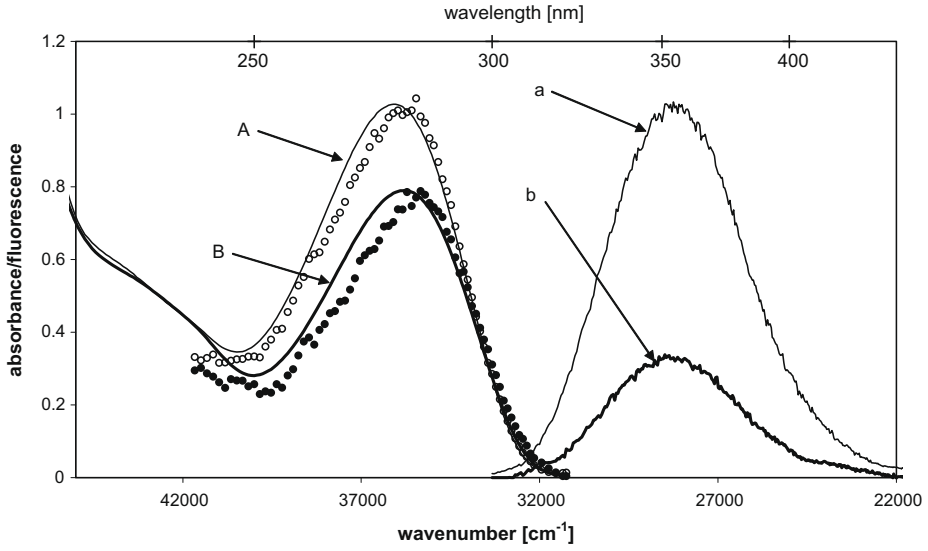


Figure 2. UV absorption (A), fluorescence emission (a), and fluorescence excitation (○ ○ ○) spectra of 8-methyl-8-azatheophylline (**4**), and UV absorption (B), fluorescence emission (b) and fluorescence excitation (● ● ●) spectra of 7-methyl-8-azatheophylline (8-azacaffeine, **3**), all in aqueous medium, with λ_{excc} 275 nm. Note that the fluorescence quantum yield of 8-azacaffeine (**3**) is about 30-fold lower than that of 8-methyl-8-azatheophylline (**4**, see Table 1). The UV absorption spectra correspond to concentrations of 100 μM, and fluorescence to ~10 μM.

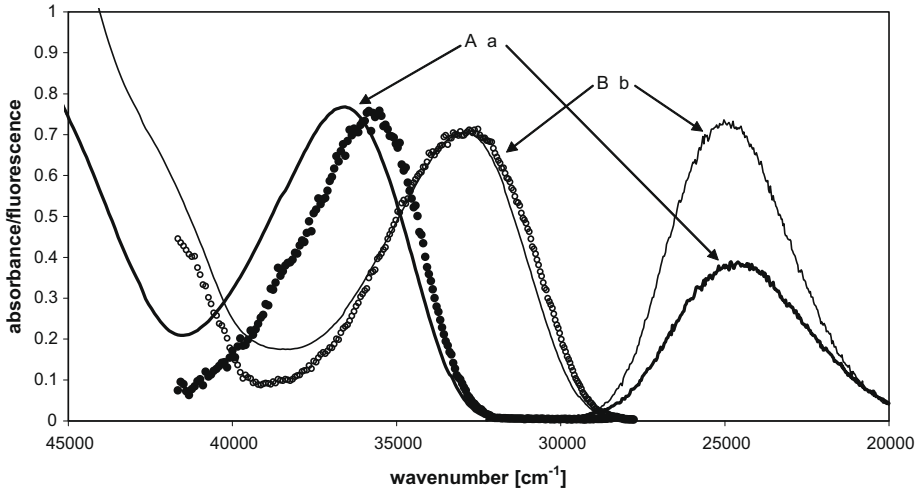


Figure 3. UV absorption (A), fluorescence emission (a) and fluorescence excitation (● ● ●) spectra of 7-methyl-8-azaXan (**6**) at pH 3; and B, b and (○ ○ ○) at pH 9. The UV absorption spectra correspond to a concentration of 100 μM.

Table 2
Estimated excited-state acidities (pK^*) for several 8-azaXan derivatives, based on the Foerster cycle

Compound, pK_a	$\nu_{00}(A^-)$ (cm^{-1})	$\nu_{00}(AH)$ (cm^{-1})	pK^*-pK	pK^*
8-Methyl- (**5**), 7.6	28,800	32,800[a]	−8.4	−0.8
7-Methyl- (**6**), 7.3	29,000	~32,600[a]	−7.5	~0
8-AzaXao (**8**), 4.65	32,000	>34,000[a]	<−4	<0.5
1,3-Dimethyl- (**2**), 4.5	~33,500[b]	33,000	+2	6.5

Note that the errors of the estimated pK^* values may be as large as ~3 pH units.[35]
[a] Estimated on the basis of UV and fluorescence spectra in methanol.
[b] Estimated on the basis of UV and corrected fluorescence spectra in methanol with ~0.4 mM triethylamine.

Foerster are only approximate, and errors of up to ~3 pH units may occur,[33,35] the general trend of the acidity changes upon excitation is quite evident from a comparison of the UV spectra of the neutral and monoanionic forms of the compounds (see Fig. 4).

The rate constants for the postulated photo-induced N(3)–H dissociation can be estimated with the Weller formula,[31] and for the compounds exhibiting $pK^* < 0$, as in 8-methyl-, and possibly 7-methyl-, 8-azaXan, they must be higher than $6 \cdot 10^{10}$ s^{-1}, leading to picosecond dissociation times and, consequently, virtually complete disappearance of the fluorescence of the neutral form in aqueous medium. Such low values for the excited state acidity (pK^*) are not uncommon among substituted heterocycles, for example, hydroxyquinoline derivatives.[36,37]

Intermolecular excited-state proton transfer reactions in purine analogues like formycin A exhibit significant deuterium isotope effects.[6] And, in fact, for 8-aza-Xan (**1**) in 0.4 mM HCl/DCl, the intensity of the long-wavelength emission band in D_2O is 63% higher than in H_2O. For the neutral form of its 8-methyl congener (**5**), the isotope effect is smaller and positive (+18%), and close to that for the anion (+23%), conceivably due to extremely rapid H/D transfer, characteristic for the very low pK^* (Table 2). By contrast, with 7-methyl-8-azaXan (**6**) and 9-benzyl-8-azaXan (**7**), we observe negative effects in 0.5 mM HCl/DCl (−27% and −21%, respectively), and positive effects in 1 mM NaOH/NaOD (+4% and +23%). This is in accord with the postulated participation of excited-state proton transfer in the decay of the S_1 state of triazole-alkylated 8-azaxanthines.

2.4. Emission of 8-azaxanthosine

The spectral properties, including emission, of 8-azaXao (**8**) are very similar, as might be anticipated, to those of 9-benzyl-8-aza-

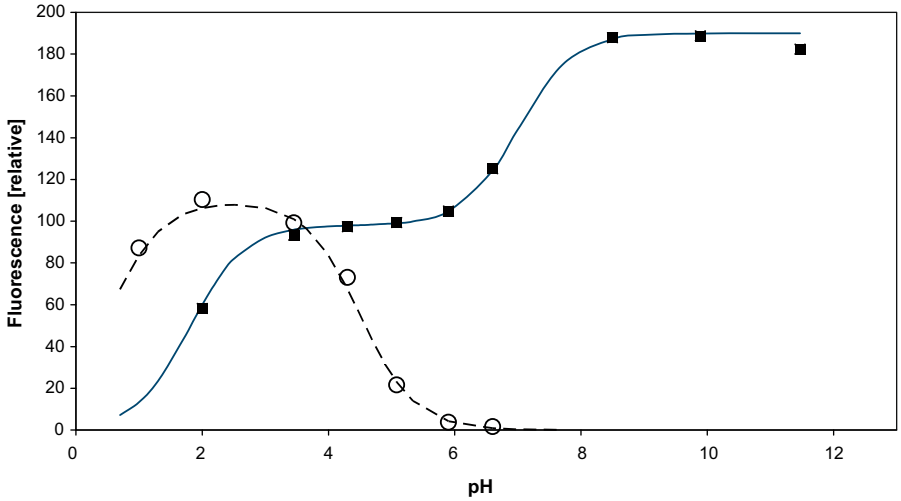


Figure 4. Fluorimetric titration of 8-azatheophylline (- - - -; **2**), monitored at 355 nm (λ_{exc} = 275 nm), and of 7-methyl-8-azaXan (——; **6**), monitored at 400 nm, with excitation at the isosbestic point, 286 nm. The fitted pK_a values are, approximately, 4.5 for 8-azatheophylline (**2**) and 7.0 for 7-methyl-8-azaXan (**6**). Note that at the lower pH range, <2, dynamic fluorescence quenching occurs, with apparent $pK \sim 0.6$ for 8-azatheophylline and 1.8 for 7-methyl-8-azaXan.

Xan (**7**), but with a somewhat lower fluorescence quantum yield (Table 1). They differ markedly from those of the parent 8-azaXan (**1**), since the latter exists as a mixture of N(7)H and N(8)H protomers at weakly acidic pH, and at pH 7 as a monoanion with negative charge on the triazole ring[18] (cf. Table 1).

Fluorimetric, as well as spectrophotometric, titrations of the nucleoside give p$K_a \sim 4.65$, somewhat lower than that for 9-benzyl-8-azaXan (5.4, Table 1), and this pK_a is most likely due to N(3)H dissociation, like in Xao.[31] Two fluorescence bands, with maxima at 365 nm and 430 nm, are observed at weakly acidic pH (pH 3–5, see Fig. 5). At more acidic pH, ~1.5, a dynamic quenching of the monoanionic fluorescence (365 nm) is observed, accompanied by an increase in intensity of the long-wavelength band (430 nm), suggesting that the latter may be due to a non-typical neutral, possibly enol, form of the compound, as described above for 9-benzyl-8-azaXan.

2.5. Fluorescence of 8-aza-9-deaza xanthine analogues

We have also observed that the neutral forms of some xanthine analogues of pyrazolo[4.3-*d*]pyrimidines (8-aza-9-deazapurines), compounds **9**, **10**, **11 (**Scheme 1) exhibit moderate, albeit readily detectable, emission (Table 1). By contrast, the anions of two of them, **9** and **11,** are intense emitters ($\phi \sim 0.15$–0.2) and, furthermore, their absorbance extends well to the red of 300 nm (Table 1) permitting of selective excitation in the presence of proteins and nucleic acids, thus rendering them particularly useful for ligand-binding studies. These compounds are easily prepared from pyrazolo[4,3-*d*]pyrimidine-7-ones by direct oxidation with bromine water.[38] The highest fluorescence quantum yields are observed for derivatives alkylated on N(8), as found for many 8-azapurine derivatives,[16,18] including non-typical nucleosides and nucleotides, ribosylated at N(8).[39]

3. Conclusions

Various alkylated 8-azaxanthine analogues exhibit strong fluorescence in aqueous medium, which has been overlooked in the chemical literature. Their fluorescence has been shown to be helpful, for example, for product identification in enzymatic ribosylation by various forms of purine-nucleoside phosphorylase, and should be useful, amongst others, in studies on the enzymes involved in caffeine biosynthesis.

Some of the investigated compounds undergo excited-state proton transfer reactions, which makes their spectral properties very sensitive to micro-environment changes, including isotope, buffer and solvent effects. They are promising candidates as fluorescent probes.

4. Experimental

UV absorption spectra were recorded on a Cary 300 instrument, and steady-state fluorescence emission on a Perkin–Elmer LS-50B, with spectral bandwidth 3–5 nm. Fluorescence quantum yields were determined relative to tryptophane (0.15). Fluorescence yields and fluorescence excitation spectra were measured in semi-micro cuvettes, with 4 mm pathlength, to avoid inner filter effect. Sample absorbance never exceeded 0.05 per 4 mm pathlength. The built-in correction curves were used to correct the excitation spectra for spectral distribution of the excitation beam.

Fluorescence decay times were measured using an Edinburgh Analytical Instruments (Edinburgh, Scotland) spectrofluorimeter model FL-900CDT with a discharge nitrogen lamp as light source (approximately 2.5 ns impulse width at half-point). Decays were fitted to single- or double-exponential models, and the proper model was selected depending on the resultant χ^2 value.

Syntheses of *N*-methyl 8-azaxanthines were according to the literature,[20,21] and their identities confirmed by comparison of UV spectral and titration data with those published earlier.[19–21] 8-azaxanthosine was synthesized enzymatically, on a milligram scale, from 8-azaxanthine, with the aid of purine-nucleoside phosphorylase II (xanthosine phosphorylase) from *E. coli* as catalyst, and 7-methyguanosine as a ribose donor, and purified by HPLC, using Beckman instrument with dC-18 Atlantis (Waters) column,[23] and gradient 0–20% of acetonitrile/water/methanol (1/2/1, v/v/v) in 0.2 M sodium acetate (pH 6.7).

Alkylated 8-aza-9-deazaxanthines (pyrazolo[4,3-*d*]pyrimidine-5,7-diones) were synthesized from the corresponding 7-ones by treatment with bromine water,[38] and addition of 3–5% DMF to increase solubility of starting compounds, at room temperature.

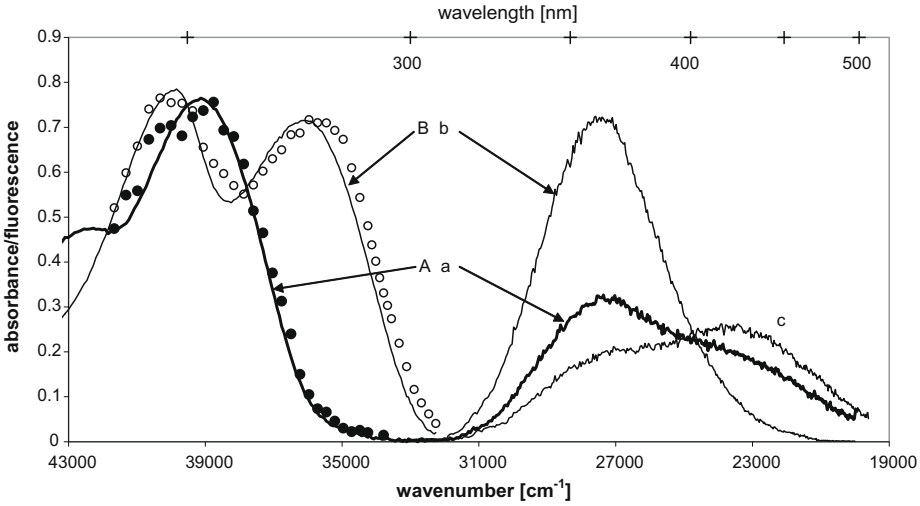


Figure 5. UV absorption (A), fluorescence emission (a), and fluorescence excitation (● ● ●) spectra of 8-azaxanthosine (**8**) in aqueous medium pH 3, and (B, b, ○ ○ ○) at pH 8. Emission was recorded with an excitation wavelength 290 nm for pH 8 and 260 nm for pH 3, at concentration <10 μM. The UV spectra are normalized to concentration 85 μM. Note that, although the UV absorption is independent of pH in the range 1–3, there is a marked change in emission from pH 3–1.5 (curve c), and lower pH, hence due to excited-state processes.

Their spectral parameters were virtually identical to those published elsewhere.[40]

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