Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Photochemistry and Photobiology A: Chemistry 179 (2006) 276–282
Journal of Photochemistry and Photobiology A:Chemistry
www.elsevier.com/locate/jphotochem

Fluorescence emission properties of 8-azaxanthine and its *N*-alkyl derivatives: Excited-state proton transfer, and potential applications in enzymology

Jacek Wierzchowski[a,*], Jerzy Sepioł[b], Daniel Sulikowski[c], Borys Kierdaszuk[d], David Shugar[d,e]

[a] *Department of Biophysics, University of Warmia and Mazury, 4 Oczapowskiego St., PL-10-719 Olsztyn, Poland*
[b] *Department of Photochemistry and Spectroscopy, Institute of Physical Chemistry, Polish Academy of Sciences, 44/52 Kasprzaka St., PL-01-224 Warsaw, Poland*
[c] *Adamed Corp., Pieńków 149, PL-05-152 Czosnów, Poland*
[d] *Department of Biophysics, Institute of Experimental Physics, University of Warsaw, Zwirki & Wigury 93, PL-02-089 Warsaw, Poland*
[e] *Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 5a Pawinskiego St., PL-02-106 Warsaw, Poland*

Received 21 May 2005; received in revised form 20 July 2005; accepted 16 August 2005
Available online 26 September 2005

Abstract

We have investigated the emission properties of 8-azaxanthine (8-azaXan) and its triazolo *N*-alkyl derivatives, with particular emphasis on *N*(8)-methyl-8-azaXan. In aqueous medium, both 8-azaXan and its 8-alkyl congener emit at 420 nm, with an unusually large Stokes' shift, and decay times of 9 and 12 ns, respectively. With the parent 8-azaXan, emission is observed over a narrow pH range (2–6), where it is partially or predominantly in its neutral form in the ground state (pK_a 4.9), whereas for 8-methyl-8-azaXan emission is observed in the pH range 2–12, hence for both the neutral and monoanionic species (pK_a 7.2). Fluorescence excitation spectra are in accord with the absorption spectra at all pH values. In anhydrous methanol, 8-azaX, in its neutral form, emits at about 335 nm. In 10% aqueous methanol, acidified to pH ∼3, two emission bands are observed, at 420 and 335 nm. With the 8-methyl derivative, dual emission at 420 and ∼340 nm is observed in anhydrous methanol, but in isopropanol and acetonitrile the 420 band virtually disappears. It is concluded that the 420 nm emission band, ascribed to the monoanionic form of 8-methyl-8-azaXan, appears in neutral or acidic media as the result of rapid deprotonation of the *N*(3)–H in the excited states of both 8-azaXan and its 8-alkyl derivative. This leads to phototautomerism in the 8-azaXan monoanion which, in the ground state, is present in the form resulting from dissociation of the triazole proton, i.e., *N*(8)–H. This proton transfer is slowed down in non-aqueous solvents, where emission from the neutral molecules (335–340 nm) is observed. The foregoing interpretation is supported by determination, with the aid of the Foerster cycle, of the excited state pK^* values, close to −0.5 for 8-methyl-8-azaXan. The strong emission of 8-azaXan and its *N*-alkyl derivatives should prove useful in studies on enzyme/ligand interactions, several examples of which are proposed. In particular, we report that 8-azaXan is a substrate for purine nucleoside phosphorylase II from *E. coli*, and show that the reaction can be monitored fluorimetrically.

Keywords: 8-Azapurines; 8-Azaxanthine; Fluorescence; Phototautomerism; Protein/ligand interactions

1. Introduction

The naturally occurring purines, and their nucleosides and nucleotides, are virtually non-fluorescent under physiological conditions. This has led to the development of two classes of fluorescent analogues which may be used as probes for studying ligand-binding reactions and kinetics in solution. One such class embraces nucleosides and nucleotides "tagged" with a

Abbreviations: 8-azaXan, 8-azaxanthine (1,2,3-triazolo[4,5-d]pyrimidine-4,6-dione); m^8-8-azaXan, 8-methyl-8-azaxanthine; m^7-8-azaXan, 7-methyl-8-azaxanthine; bz^9-8-azaXan, 9-benzyl-8-azaxanthine; PNP, purine nucleoside phosphorylase

* Corresponding author. Tel.: +48 89 5233406; fax: +48 89 523 4320.
E-mail address: jacek.wie@uwm.edu.pl (J. Wierzchowski).

1010-6030/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jphotochem.2005.08.027

fluorescent moiety chemically linked to either the sugar or phosphate [1–3]. These offer a variety of desirable features, including monitoring of single-molecule fluorescence; but their application is frequently limited by steric effects due to bulkiness of the attached fluorophore.

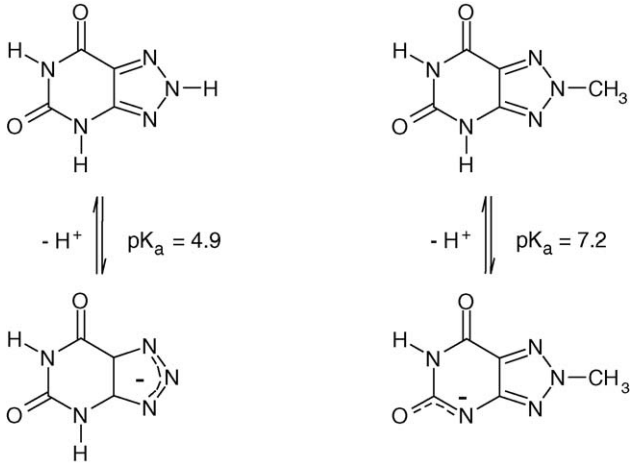
Also widely profited from are a limited number of fluorescent structural analogues of the natural purines, including 2-aminopurine [2], 2,6-diaminopurine, 1,6-ethenoadenosine and 2,3-ethenoguanosine [4], lin-benzopurines [5], and formycin A (a close structural analogue of adenosine [6,7]). It is, of course, necessary to delineate their spectral behaviour in solution, frequently dependent on acid–base properties and both ground- and/or excited-state tautomerism [4,6,8–10].

Although the synthesis and physico-chemical properties of 8-azapurines were long ago extensively reviewed by Albert [11], very little attention appears to have been directed to the fact that a number of these exhibit intrinsic fluorescence, e.g. the neutral form of 8-azaadenosine (8-azaAdo), the monoanions of 8-azaguanosine (8-azaGuo) and 8-azainosine (8-azaIno), the neutral form of 8-azaguanine (8-azaGua) [12,13], all of which are substrates/inhibitors of bacterial and/or mammalian purine nucleoside phosphorylases (PNP) [12,14].

Our ongoing studies on the properties of 8-azapurines have profited from the intrinsic emission of 8-azaGua to investigate its interaction with calf spleen PNP [14,15]. This, in turn, led to the finding that 8-azaxanthine (8-azaXan, see Scheme 1), the product of enzymatic deamination of the known cytotoxic agent 8-azaGua [16], and an inhibitor of several key enzymes involved in purine metabolism [17–19], reveals strong intrinsic fluorescence in weakly acid medium, potentially useful in enzymological studies.

Nubel and Pfleiderer [20] first reported the synthesis and some properties of 8-azaXan and its various *N*-methyl derivatives and, in particular, the marked acidity of the parent 8-azaXan which, in neutral aqueous medium, exists as a monoanion (pK_a 4.95), resulting from dissociation of the triazole proton (cf. [11]). Prototropic tautomerism of 1,3-dimethyl-8-azaXan (8-azatheophylline) was subsequently investigated by L'abbe et al. [21] by means of ^{13}C and ^{15}N NMR spectroscopy, with results pointing to predominance (80%) of the *N*(8)–H form in DMSO solution, the sole form found for the parent neutral monohydrate of 8-azaXan, and other 8-azapurines, in the crystalline state [22].

We here report on the fluorescence emission properties of 8-azaXan, and its triazolo *N*-alkyl derivatives, to determine which of the prototropic tautomers is responsible for its emission in weakly acidic medium, essential for interpretation of its behaviour as a fluorescent probe in ligand-binding studies in solution.



Scheme 1. 8-Azaxanthine (left) and 8-methyl-8-azaxanthine (right), shown in their most stable ground state neutral (upper) and monoanionic (lower) forms.

2. Materials and methods

2.1. General

8-Azaxanthine (from ICN Chemicals) was recrystallized as the monosodium salt. Its triazolo *N*-alkyl derivatives were synthesized as described below. Solvents (UV spectroscopy grade) were from Merck, and water was filtered through a Millipore Milli-Q system. Purified PNP-II from *E. coli* was a gift of Dr. Gert Dandanell [23].

Fluorescence spectra were run on a Perkin-Elmer LS-50B fluorimeter, equipped with a pulsed xenon light source, and with typical resolution of 3–5 nm. UV absorption spectra were run on a Cary 319. Fluorescence excitation spectra were corrected for lamp spectral characteristics and for the inner filter effect. Quantum yields were measured relative to tryptophan (0.15), additionally checked relative to quinine sulfate (0.55).

Fluorescence decay times were measured using an Edinburgh Analytical Instruments (Edinburgh, Scotland) spectrofluorimeter model FL-900CDT with a discharge hydrogen 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.

2.2. Synthetic procedures

The three alkyl derivatives of 8-azaXan were prepared essentially according to known procedures, with some modifications. The starting point for all preparations was 1-benzyl-5-amino-1,2,3-triazolo-4-carboxamide, synthesized according to Hoover and Day [24], from benzyl azide and cyanoacetamide. When fused with urea at 175 °C for 2.5 h as elsewhere described [25], this compound afforded the hitherto unknown 9-benzyl-8-azaXan, with ca. 70% yield. Identity of the final product was confirmed by MS ESI(+) (246 [MH+]), elementary analysis: (calculated for $C_{11}H_{11}N_5O_2$: C, 53.87%; H, 4.52%; N, 28.56%. Found: C, 53.89%; H, 4.55%; N, 28.54%), m.p. 217 °C. Its UV spectrum, as a function of pH, was virtually identical with that reported by Nubel and Pfleiderer [20] for 9-methyl-8-azaXan.

N(7)-Methyl-8-azaXan was obtained from the starting 1-benzyl triazole, by alkylation with tosyl methylate in DMSO at 150 °C [26], followed by catalytic debenzylation (10 atm H_2 with 10% palladium on charcoal, see [20]), and fusion with urea. *N*(8)-Methyl-8-azaXan was synthesized from 5-amino-

Table 1
Ionization constants (pK_a values) and spectral parameters for neutral and ionic forms of 8-azaxanthine and some *N*-methyl derivatives

Compound	pK_a	Form (pH)[a]	UV absorption		Fluorescence	
			λ_{max} (nm)	ε_{max} [M^{-1} cm^{-1}]	λ_{max} (nm)	ϕ
8-azaXan	4.96, 10.2 4.66; 9.79[b]	n (3.0)	263	6500	420	~0.21[c]
		ma (7.0)	265	8500	–	<0.005
		da (12.3)	285	6500	–	<0.005
N(9)-Methyl-	5.36[b]	n (2.0)	255	9100	365,430[d]	0.075[d]
		ma (7.0)	278	7600	365[d]	0.06[d]
N(8)-Methyl-	7.04[b], 13.4	n (4.0)	272	8700	420	0.50
		ma (11.0)	299	6450	420	0.60
N(7)-Methyl-	7.22[b]	n (3)	273	6030	400, 430	0.13
		ma (11.3)	304, 302[b]	5750	401	0.23
N(1)-Methyl-	4.67, 9.85[b]	n	263	6300	nd	–
		ma	265	8100	nd	–
N(3)-Methyl-	4.4, 11.37[b]	n	270	5400	nd	–
		ma	268	7100	nd	–
N(8),*N*(1)-Dimethyl-	7.7[b]	n	274	8500	nd	–
		ma	300	6600	nd	–
N(8),*N*(3)-Dimethyl-	9.16[b]	n	277	9100	nd	–
		ma	282	10000	nd	–

Data from Albert [11]; Nubel and Pfleiderer [20], and the present work. Fluorescence quantum yields are relative to tryptophan (0.15), additionally checked against quinine sulfate (0.55).
[a] n: neutral from; ma: monoanion; da: dianion.
[b] Data from Nubel and Pfleiderer [20], pK_a values determined electrochemically.
[c] Value obtained with λ_{exc} 275 nm in 5 mM acetate buffer, pH 4.2 (this work).
[d] Data for 9-benzyl derivative.

1,2,3-triazole-4-carboxamine as described by Albert [25,27]. The starting compound was obtained from the benzyl derivative by treatment with concentrated sulfuric acid for 4 days at room temperature, with ~70% yield. The spectral parameters for both 7-methyl and 8-methyl-8-azaXan were identical to those reported by Nubel and Pfleiderer [20], and are listed in Table 1. Their purity, assessed by HPLC/MS, was virtually 100%.

3. Results and discussion

3.1. Fluorescence of 8-azaxanthine

8-azaXan is virtually nonfluorescent in neutral aqueous medium, but below pH 6 it begins to emit, with strong fluorescence, centered at about 420 nm (Fig. 1). A striking feature of this emission is its unusually large Stokes' shift (up to 14,200 cm^{-1}). By contrast, when 8-azaXan was dissolved in anhydrous methanol, acidified with ~1 mM glacial acetic acid to ensure the presence of the neutral form of the compound, the fluorescence maximum was shifted to ~335 nm (Fig. 1). In 10% aqueous methanol, acidified to pH ~3, dual fluorescence is observed, with maxima at 335 and 420 nm (not shown), and an identical excitation spectrum for both bands. This spectral behaviour, in particular the dual emission, is typical for excited-state molecular rearrangements [28], and the strong influence of water points to participation of excited-state proton transfer.

8-azaXan has three mobile protons and, additionally, several basicity centers, offering various possibilities for excited-state proton dissociation or protonation. Ground-state tautomerism of this compound has been partially resolved both experimentally and theoretically [20–22]. Like the parent neutral Xan, a 2,6-diketo structure prevails, with the triazole proton located primarily at *N*(8), as evidenced by the NMR spectra of the 1,3-dimethyl derivative in DMSO [21], and the possibility to tautomerize to *N*(7). This proton is also the most acidic (pK_a

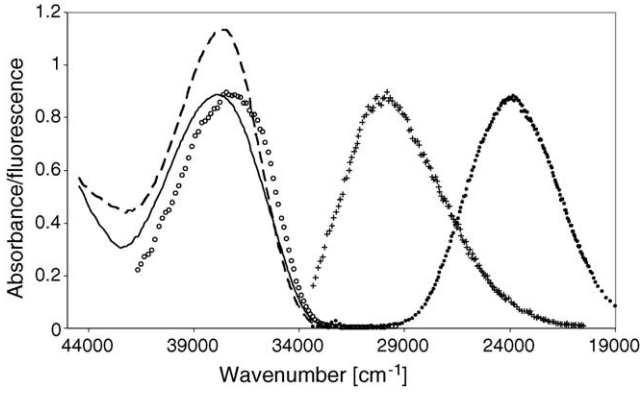


Fig. 1. UV absorption and fluorescence spectra of 8-azaXan in various conditions. Left-hand side: (–), UV absorption at pH 2; (--), UV absorption at pH 7; (○), fluorescence excitation spectrum at pH 4. Right-hand side: (●), fluorescence emission in 1 mM HCl; (+), fluorescence emission in anhydrous methanol.

4.95), fully confirmed by UV spectra and acid–base properties of *N*-methyl derivatives [20].

A possibility of the excited-state proton migration can be evaluated on the basis of the Foerster cycle [28], allowing calculation of pK^* by comparison of the UV spectra of the acidic and basic forms of the compound. Since the long-wavelength maxima of the neutral and monoanionic forms of 8-azaXan are close (see Fig. 1), we conclude that pK^* for *N*(8)–H must be close to the ground pK_a value (4.9), and therefore *N*(8)–H is not likely to migrate upon excitation of the molecule.

3.2. N-Alkyl derivatives of 8-azaxanthine

We then turned to an examination of *N*(8)-methyl-8-azaXan (m^8-8-azaXan), a compound modeling the major tautomer of the neutral 8-azaXan [22], but lacking the ability to form an anion with a negative charge on the triazole ring (Scheme 1). It is much less acidic than 8-azaXan, with a pK_a of 7.2 for dissociation of the *N*(3)H, and a second pK_a ~13.4 for the *N*(1)H [11,20]. This order of dissociation is inferred from a comparison of the UV spectra of the N_1,N_8-dimethyl and N_3,N_8-dimethyl derivatives in their monoanionic forms with the spectrum of the m^8-8-azaXan monoanion (cf. [20] and Table 1).

The methylated compound is also intensely fluorescent at 420 nm, but, unlike 8-azaXan, its emission is observed over a very broad pH range, from pH ~2 to ~12 (cf. Figs. 2 and 3). The fluorescence excitation spectrum is virtually identical to the UV absorption spectrum in this pH range (cf. Fig. 2). This situation is somewhat reminiscent of N_1,N^6-ethenoadenosine, which exhibits unchanged fluorescence in neutral and acidic media despite its being protonated in the ground state, a fact interpreted as a fast proton dissociation after excitation of the cation [4]. By analogy, we postulate rapid deprotonation of *N*(3)–H in the excited state of m^8-8-azaXan to interpret its fluorescence spectrum in weakly acidic media.

It should be noted that deprotonation of *N*(3)–H is in this case much more energetically favorable than dissociation of *N*(1)–H, as concluded from a comparison of the UV spectra

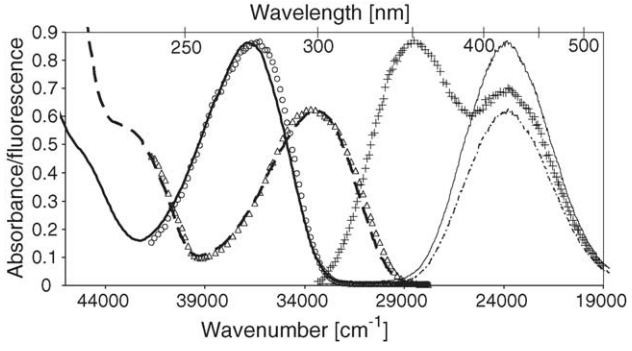


Fig. 2. UV absorption and fluorescence spectra of N8-methyl-8-azaxanthine (100 μM) in various conditions. Left-hand side: (–), UV absorption at pH 3; (--), UV absorption at pH 11; (○), fluorescence excitation spectrum at pH 3; (△), fluorescence excitation spectrum at pH 11. Right-hand side: (-·-·-), fluorescence emission in 1 mM HCl; (—), fluorescence emission at pH 11; (+), fluorescence emission in methanol. Fluorescence spectra are normalized to the absorption band of lowest energy.

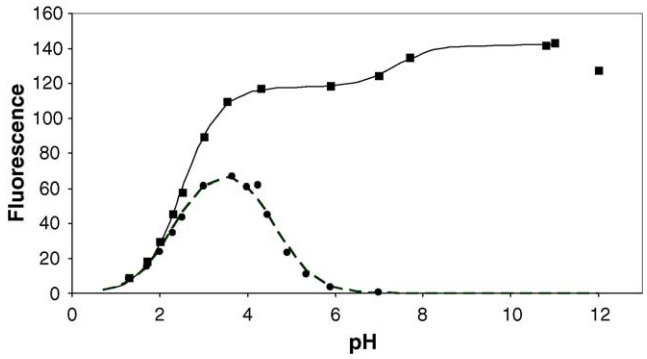


Fig. 3. Fluorescence intensity of 8-azaX (•) and m^8-8-azaXan (■) monitored at 420 nm, as a function of pH. Fluorescence was measured in 5 mM acetate and phosphate buffers (pH 3.6–8.5) and in dilute HCl and KOH (extreme pH values). Excitation wavelengths were 275 nm for 8-azaXan and 287 nm (isosbestic point) for m^8-8-azaXan. The fitted pK values for 8-azaXan are 2.3 and 4.65, and for m^8-8-azaXan 2.5 and 7.4.

of the dimethyl derivatives in alkaline media (cf. Table 1). Since the batochromic shift between the neutral and monoanionic forms is ~26 nm for N_8,N_1-dimethyl-8-azaXan, compared to only 5 nm for the N_8,N_3-dimethyl derivative, $\Delta pK = pK_a - pK^*$ must be much greater for the *N*(3)–H than for the *N*(1)–H, indicating that pK^* for the latter is higher by at least 4–6 pH units (cf. Table 1). Therefore, the excited-state dissociation of *N*(1)–H, even if possible energetically, certainly cannot kinetically compete with the *N*(3)–H.

In non-aqueous solvents, such as anhydrous methanol, isopropanol and acetonitrile, an intense fluorescence band at ~340 appears, which must be ascribed to the neutral form of the molecule. But, in contrast to the parent 8-azaXan, the 420 nm band is still present in anhydrous methanol (Fig. 2), and to much lesser extent, in isopropanol, disappearing completely in acetonitrile (data not shown). This indicates that some non-aqueous solvents, like anhydrous methanol, albeit poorer proton acceptors than water, may occasionally also enable excited-state proton transfer.

Deprotonation of the excited molecule during its lifetime, with water as the proton acceptor, is possible only if pK^* is sufficiently low [28]. This value for the *N*(3)–H of m^8-8-azaXan is readily estimated, using the Foerster cycle (cf. [28]):

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

where ν_{00A} and ν_{00AH} are the wave numbers of the 0–0 transitions of the deprotonated and protonated forms, respectively. The large red shift of the monoanionic UV absorption relative to that of the neutral molecule (cf. Fig. 2 and Table 1) points to a marked difference between pK and pK^*. Since the fluorescence of the neutral molecule is not observed in water, we approximate it by its fluorescence observed in anhydrous methanol. With this assumption, and estimation of ν_{00} as a crossing point of the normalized lowest absorption and fluorescence bands [28], we obtain $\nu_{00A} - \nu_{00AH}$ ~3800 cm^{-1} (cf. Fig. 2) and, consequently, pK^* close to −0.5. This value is amongst the lowest values for a pK^* reported in the literature [28].

The low pK^* value allows us to estimate the first-order rate constant for proton transfer to a water molecule as ~3-fold

greater than the diffusion-controlled protonation rate constant at pH 0, which is $\sim 5 \times 10^{10}\ s^{-1}$ (cf. [29,30]). The resultant proton transfer rate, $\sim 1.5 \times 10^{11}\ s^{-1}$, is much higher than the estimated rate constant for radiative decay of the neutral molecule ($<10^{9}\ s^{-1}$), and explains the observed absence of emission of the neutral molecule in aqueous medium.

There is also some evidence for excited-state-proton transfer processes in the other two fluorescent *N*-alkyl derivatives of 8-azaXan, the m^7-8-azaXan, and bz^9-8-azaXan (cf. Table 1), but the general picture for these two is much more complex than that for m^8-8-azaXan, and requires further investigation. The pK^* value for the m^7-8-azaXan, evaluated by the methodology analogous to that applied for the *N*(8)-methyl derivative (see above), was found to be $\sim 0.0 \pm 1.0$ (the uncertainty results from the low fluorescence yield of the 340 nm band in methanol), again confirming both energetic and kinetic feasibility of proton photodissociation. Interestingly, the lowest excited singlet states of bz^9-8-azaXan and its monoanion are destabilized relative to the other two alkyl derivatives (see Table 1), so that the *N*(9)–H tautomer, dominating in natural purines [9], is apparently absent in both ground and excited states of 8-azaXan.

3.3. Excited-state proton transfer and phototautomerism in 8-azaxanthine and other purine analogues

Demonstration of excited-state proton dissociation in m^8-8-azaXan leads to a simple interpretation of the parent 8-azaX fluorescence in weakly acidic media, as originating from the anionic phototautomer with the *N*(1)–H,*N*(8)–H structure, and the negative charge located either at *N*(3) or delocalized in the *N*(3)–O^2 enolate, as shown in Scheme 2:

In non-aqueous media, proton-transfer is hindered, and fluorescence of the neutral molecule at 335–340 nm becomes dominant in both 8-azaXan and m^8-8-azaXan (cf. Figs. 1 and 2). The absence of phototautomeric emission in neutral aqueous medium, where 8-azaXan exists as a monoanion, may be attributed to the very short excited-state lifetime of the major monoanionic form (i.e. that with the negative charge on the triazole ring), as inferred from its low fluorescence quantum yield.

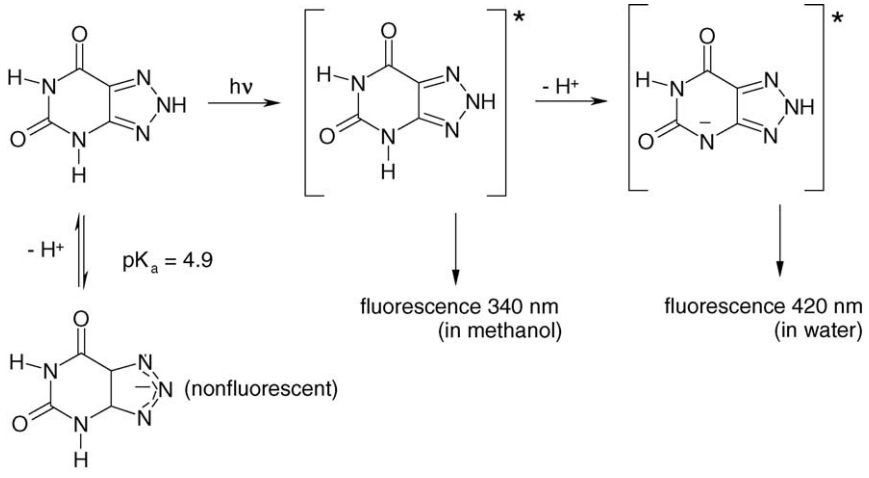
The foregoing phenomenon of phototautomerism is not unusual amongst purine analogs, as demonstrated long ago for pyrazolopyrimidines [6,8] and later for other systems [31], and postulated for other purine analogues [32,33]. However, for purine-like systems the energetic possibility of phototautomerism is not a sufficient condition for this to occur, since two-step tautomerisation, even in aqueous solvents, is typically much slower than the decay of excited singlet states of these molecules [34]. Only in very favorable conditions, as for the 7-azaindole dimer or its complexes with ethanol, may this process occur via a concerted double-proton transfer, with picosecond kinetics, leading to phototautomer emission [35–37]. Phototautomerization is, however, much easier to observe if the first of the two required steps occurs in the ground state, i.e. by prior protonation or deprotonation of the molecule, as in the case of formycin A [6] and 8-azaXan.

3.4. Decay times and dynamic quenching of fluorescence

In more acidic media (pH < 3) strong fluorescence quenching is observed for both 8-azaXan and m^8-8-azaXan (Fig. 3). To analyze its origin, we conducted fluorescence decay measurements, with results displayed in Fig. 4. It is clear that acidic quenching of 8-azaXan fluorescence is dynamic, while that observed at pH $\sim$5 (i. e. close to the ground-state pK_a) is static. As expected, the fluorescence lifetime of the phototautomeric emission of 8-azaXan is long ($\sim$9 ns), and the lifetime of the neutral molecule in the absence of phototautomerism (i.e. in methanol) is $\sim$0.9 ns, which is still much longer than the estimated proton transfer rate in water (see Section 3.2).

Decay times for m^8-azaXan (Fig. 4) are slightly longer, $\sim$12 ns, and the apparent pK fitted from the lifetime data (2.4) is close to that obtained from steady-state titration (2.65). Again, the ground-state pK_a value has no influence on fluorescence decays, which can be explained by very rapid proton transfer from neutral excited m^8-8-azaXan to a water molecule.

The described dynamic fluorescence quenching in the acidic region, most likely due to excited-state reprotonation with H_3O^+ as the proton donor, is also typical for other purine-like sys-



Scheme 2. Proposed origin of the 420 nm and 335 nm fluorescence bands of 8-azaXan in aqueous and methanolic media.

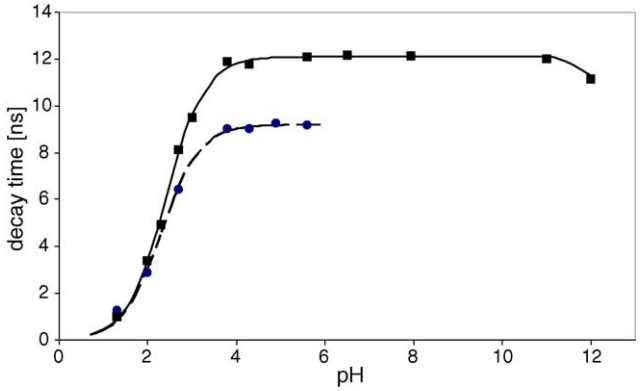


Fig. 4. Fluorescence decay times for 8-azaXan (●) and m_8-8-azaXan (■), monitored at 420 nm, at various pH. Excitation was at 280 nm for 8-azaXan and 287 nm for m^8-8-azaXan. Buffer concentrations did not exceed 5 mM, except for extreme pH values. All decay curves were analyzed as single exponential decays, with $\chi^2 < 1.2$ in all instances. The apparent pK for dynamic quenching is 2.3 for 8-azaXan and 2.4 for m^8-8-azaXan.

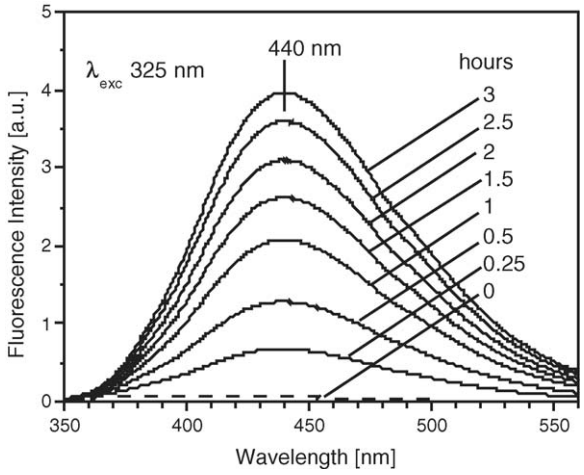


Fig. 5. Time-course of the reverse synthetic reaction of *E. coli* PNP-II with 8-azaXan as a substrate, in the presence of 2 mM α-D-ribose-1-phosphate. The reaction was carried out in 50 mM Hepes (pH 7) at 25 °C, and the fluorescence emission of the nucleoside product was selectively excited at 325 nm.

tems undergoing proton dissociation in their excited states in weakly acidic media, e.g. formycin A [6], 4-aminopyrazolo[3,4-d]pyrimidine [8] and N_1,N^6-ethenoadenosine [4]. It must be stressed that, in the case of 8-azaXan, reprotonation most certainly does not occur at $N(3)$, since the low pK^* (−0.5) precludes this, and the quenching at pH < 3 is not accompanied by an increase in emission of the neutral molecule at 335 nm. This fact illustrates some fundamental difficulty with application of the pK^* concept for multi-basic compounds like purines: due to lack of thermodynamic equilibrium in the singlet excited states, no "overall", or thermodynamic, pK^* value can be estimated, and values calculated from the Foerster cycle are rather "microscopic" pK^* values for individual basicity centers, and may be distinct for each individual center.

4. Conclusions and potential applications

The unusual, and strong, fluorescence of 8-azaXan and its *N*-alkyl derivatives should prove useful for the studies of protein–ligand interactions, since these are expected to markedly influence kinetics of excited-state proton transfer, and, consequently, the emission spectra of the bound ligands. Perspectives include, amongst others, investigations of adenosine receptors, for which some 8-azaxanthine derivatives are known as potent antagonists [38], and of urate oxidase, for which 8-azaXan (a structural analogue of the substrate, urate) is a good competitive inhibitor, employed for studying the mode of interaction of the enzyme with its substrate in the solid state [19].

Furthermore, since Xan is a substrate for the PNP from mammalian sources, but not for the PNP-I from *E. coli* (product of the *deoD* gene) [39,40], we examined also 8-azaXan as a potential substrate. We have found, in preliminary studies, that 8-azaXan is not a substrate for the foregoing enzymes. In striking contrast, both Xan and, to a lesser extent, 8-azaXan, are substrates for the *E. coli* PNP-II (product of the *xapA* gene [23]). The course of conversion of 8-azaXan to its nucleoside by this enzyme, although relatively slow, may be readily monitored fluorimetrically, as shown in Fig. 5, at excitation wavelengths above 300 nm, where the parent 8-azaXan does not absorb, and the emission originates only from the nucleoside product. This nucleoside, in its neutral form, exhibits a Stokes' shift ∼13,500 cm^{-1}, comparable to that for 8-azaXan and its *N*-alkyl derivatives (see Section 3.1 and Table 1), suggesting, that similar proton-transfer phenomena occur also at the nucleoside (and, probably, nucleotide) level, thus extending the area of potential applications to investigations on nucleic acids.

Acknowledgement

This work profited from a grant #3P04A 024 25 from the Ministry of Science and Informatics (MNiI)

References

[1] D.M. Jameson, J.F. Eccleston, Methods Enzymol. 278 (1997) 363–390.
[2] D.P. Millar, Curr. Opin. Struct. Biol. 6 (1996) 322–326.
[3] T. Ha, Curr. Opin. Struct. Biol. 11 (2001) 287–292.
[4] N.J. Leonard, CRC Crit. Rev. Biochem. 15 (1984) 125–199 (and references cited therein).
[5] N.J. Leonard, Biopolymers 24 (1985) 9–28.
[6] J. Wierzchowski, D. Shugar, Photochem. Photobiol. 35 (1982) 445–458.
[7] B. Kierdaszuk, A. Modrak-Wójcik, J. Wierzchowski, D. Shugar, Biochim. Biophys. Acta 1476 (2000) 109–128.
[8] J. Wierzchowski, M. Szczęśniak, D. Shugar, Z. Naturforsch. 35c (1980) 878–889.
[9] M. Wilson, P.R. Callis, Photochem. Photobiol. 31 (1980) 323–327.
[10] J. Sepiol, Ph.D. thesis, Warsaw University, 1978.
[11] A. Albert, Adv. Heterocycl. Chem. 39 (1986) 117–178.
[12] J. Wierzchowski, B. Wielgus-Kutrowska, D. Shugar, Biochim. Biophys. Acta 1290 (1996) 9–17.
[13] F. Seela, I. Munster, U. Loechner, H. Rosemeyer, Helv. Chim. Acta 81 (1998) 1139–1155.
[14] J. Wierzchowski, A. Bzowska, K. Stępniak, D. Shugar, Z. Naturforsch. 59c (2004) 713–725.
[15] J. Wierzchowski, K. Stępniak, A. Bzowska, D. Shugar, Nucleosides, Nucleotides Nucl. Acids, in press.

[16] S. Ito, T. Takeshi, H. Mori, A. Teruo, Clin. Chim. Acta 115 (1981) 135–144.
[17] R. Perez-Vicente, J.M. Alamillo, J. Cardenas, M. Pineda, Biochim. Biophys. Acta 1117 (1992) 159–166.
[18] N. Colloc'h, M. el Hajji, B. Bachet, G. L'Hermite, M. Schiltz, T. Prange, B. Castro, J.P. Mornon, Nat. Struct. Biol. 4 (1997) 947–952.
[19] P. Retailleau, N. Colloc'h, D. Vivares, F. Bonnete, B. Castro, M. El-Hajji, J.P. Mornon, T. Prange D60 (2004) 453–462.
[20] G. Nubel, W. Pfleiderer, Chem. Ber. 98 (1965) 1060–1073.
[21] G. L'abbe, M.-A. Persoons, S. Toppet, Magn. Res. Chem. 25 (1985) 362–364.
[22] M.P. Sanchez, M.A. Romnero, J.M. Salas, D.J. Cardenas, J. Molina, M. Quiros, J. Mol. Struct. 344 (1995) 257–264.
[23] G. Dandanell, R.H. Szczepanowski, B. Kierdaszuk, D. Shugar, M. Bochtler, J. Mol. Biol. 348 (2005) 113–125.
[24] J.R. Hoover, A.R. Day, J. Am. Chem. Soc. 78 (1956) 5832–5836.
[25] A. Albert, H. Taguchi, J. Chem. Soc. Perkin I (1972) 449–456.
[26] A. Albert, J. Chem. Soc. C (1968) 344–347.
[27] A. Albert, J. Chem. Soc. C (1968) 2076–2083.
[28] B. Valeur, Molecular Fluorescence, Wiley-VCH, Weinheim, 2002, pp. 99–109.
[29] A. Weller, in: G. Porter (Ed.), Progr. React. Kinet., vol. I, Pergamon Press, Oxford, 1961, pp. 189–203.
[30] J.F. Ireland, P.A.H. Wyatt, Adv. Phys. Org. Chem. 12 (1976) 131–221.
[31] G. Wenska, B. Skalski, M. Insinska, S. Paszyc, R.E. Verrall, J. Photochem. Photobiol. A 108 (1997) 135–142.
[32] S.K. Srivastava, C. Mishra, J. Theor. Biol. 93 (1981) 655–666.
[33] C. Santhosh, P.C. Mishra, Spectrochim, Spectrochim. Acta Part A: Mol. Spectrosc. 47 (1991) 1685–1693.
[34] G. Dodin, O. Bensaude, J.-E. Dubois, J. Am. Chem. Soc. 102 (1980) 3897–3899.
[35] H. Bulska, A. Grabowska, B. Pakuła, J. Sepioł, J. Waluk, U.P. Wild, J. Luminescence 29 (1984) 65–81.
[36] J. Catalan, J.C. del Valle, M. Kasha, Chem. Phys. Lett. 318 (2000) 629–636.
[37] S. Takeuchi, T. Takahara, Chem. Phys. Lett. 347 (2001) 108–114.
[38] P. Franchetti, L. Messini, L. Cappellacci, M. Grifantini, A. Lucacchini, C. Martini, G. Senatore, J. Med. Chem. 37 (1994) 2970–2975.
[39] G. Stoychev, B. Kierdaszuk, D. Shugar, Eur. J. Biochem. 269 (2002) 4048–4057.
[40] E. Kulikowska, B. Kierdaszuk, D. Shugar, Acta Biochim. Pol. 51 (2004) 493–531.