

Pyrazolo[4,3-*e*][1,2,4]triazines: Purine Analogues with Electronic Absorption in the Visible Region

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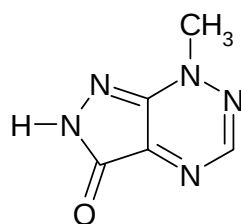
Abstract: Synthesis of several pyrazolo[4,3-*e*][1,2,4]-triazines is described. The absorption spectrum of some 5-substituted derivatives was found to extend to the visible region. These compounds were found to inhibit some enzymes of purine metabolism, like xanthine oxidase or bacterial purine-nucleoside phosphorylase with K_i values in the 10^{-3} – 10^{-5} M range.

Keywords: Pyrazolo[4,3-*e*][1,2,4]triazines, purine analogues, enzyme inhibitors.

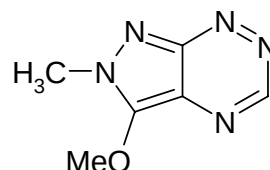
Introduction

Modified purine nucleosides and their analogues have found numerous applications in biological research and pharmacology [1-3]. In particular, the isomeric pyrazolopyrimidines and triazolopyrimidines (8-azapurines) exhibit favourable spectroscopic and biochemical properties and have potential for use in cancer and viral chemotherapy [3-6]. It has been found recently that another class of naturally occurring purine analogues produced by *Pseudomonas fluorescens* var. *pseudoiodinium* and *Nostoc spongiaeforme* such as pseudoiodinine and nostocine A (Figure 1) display even more interesting spectral characteristics, i.e. their electronic absorption spectra extend well to the visible region [7,8].

Figure 1



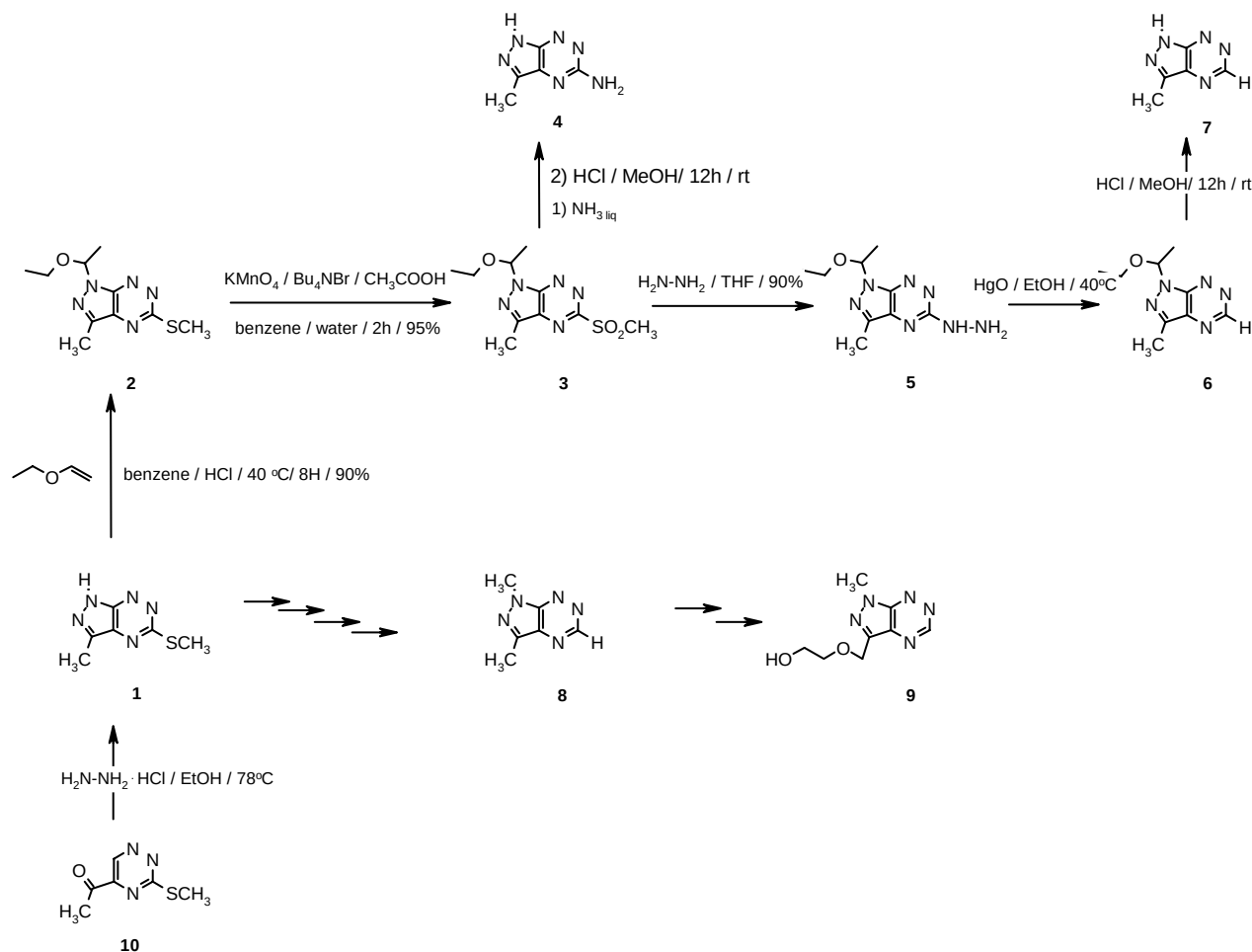
Nostocine A



Pseudoiodinine

Recent advances in nucleophilic substitution of hydrogen in heteroaromatics [9] and their successful application to the preparation of functionalized 1,2,4-triazines [10,11] prompted us to exploit this approach to make the N-unsubstituted pyrazolo[4,3-c][1,2,4]triazine **1** [12]. In this paper some derivatives of such a system (compounds **2-9**) have been synthesized from the common intermediate **1**, as depicted in Scheme 1. Furthermore we show that these compounds both exhibit interesting spectral behaviour and also interact with enzymes of purine metabolism, and therefore may find applications as spectroscopic probes.

Scheme 1



Results and Discussion

Chemistry

The starting material 3-methyl-5-methylsulfanyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazine (**1**) was synthesized from 5-acetyl-3-methylsulfanyl-1,2,4-triazine (**10**) [13] in a one-pot reaction by condensation with hydrazine hydrochloride, followed by acid-promoted ring closure of the resulting intermediate. According to expectations, compound **1**, bearing an NH-fragment, appeared to be unreactive towards nucleophilic displacements and attempts to perform direct nucleophilic substitutions of the methylsulfanyl group with either ammonia or hydrazine failed.

To avoid these problems another possibility to synthesize *N*-unsubstituted pyrazolo[4,3-*e*][1,2,4]triazines **4** and **7** is to use a *N*-protecting group. We decided to exploit ethyl vinyl ether as a new protecting group for NH-pyrazoles [14]. Exposure of pyrazolo[4,3-*e*][1,2,4]triazine **1** to ethyl vinyl ether in benzene for 8 hours at 40°C in the presence of traces of concentrated HCl gave the intermediate **2** in 90% yield. This was isolated by chromatography and treated with potassium manganate (VII) under phase transfer catalytic conditions at room temperature for 1h to give sulfone **3** in nearly quantitative yield.

Compound **3** was next reacted with liquid ammonia at -33°C. The product was cleanly deprotected with concentrated HCl in methanol at room temperature to give the corresponding amino compound **4**, as a yellowish crystalline solid, in 80% yield. Similarly, the treatment of **3** with anhydrous hydrazine in tetrahydrofuran provides 5-hydrazino derivative **5** in 90% yield. Oxidation of the latter with yellow mercury (II) oxide results in replacement of the hydrazine group by hydrogen giving compound **6**. Deprotection of **6** in methanol with concentrated HCl at room temperature for 12 hours gave the 5-unsubstituted pyrazolo[4,3-*e*][1,2,4]triazine **7** in excellent yield (see Scheme 1).

Compound **8** was synthesized in four steps from 3-methyl-5-methylsulfanyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazine (**1**) by methylation with iodomethane, oxidation with potassium manganate (VII), hydrazinolysis with anhydrous hydrazine and treatment of the resulting 5-hydrazino-1,3-dimethyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazine with yellow mercury (II) oxide. Bromination of **8** with *N*-bromo-succinimide followed by nucleophilic displacement of bromide in the resulting C-bromomethyl derivative by ethylene glycol anion gave aza analogue of acylopurine nucleoside **9**.

Spectral properties

In agreement with previous reports [7, 8, 15], the electronic absorption spectra of 1-*H*-pyrazolo[4,3-*e*][1,2,4]triazines differ very markedly from those of the analogous purines. Low-yield fluorescence in the visible region (ca. 500 nm) was observed for some compounds in neutral aqueous medium. The basic spectral characteristics of the synthesized compounds are summarized in Table 1.

Table 1. Spectral parameters (electronic absorption and/or fluorescence) for selected pyrazolo[4,3-e]-triazines in aqueous solution. Conditions: 50 mM phosphate buffer (pH 7) or 10 mM KOH (pH 12).

Compound	pH	$\lambda_{\max}$ [nm]	$\epsilon_{\max}$ [cm ⁻¹ M ⁻¹]	fluorescence $\lambda_{\max}$ [nm]
7	7	346	2270	480w ^a
	12	418	1450	nf ^b
4	7	412	1570	490w
	12	453	780	nf
1	7	408	2300	nf
	12	438	1370	nf
9	7 - 12	356	2460	490w
8	7 - 12	370	~2400	nd ^c

^aweak fluorescence ($\phi \sim 0.01$)

^bno fluorescence detected ($\phi < 0.001$)

^cnd – not determined

Enzymatic assays

We have examined several purine metabolism enzymes to check if any of these effectively interact with the new compounds. It was found that enzymatic phosphorolysis of m⁷Guo [16], catalyzed by *E. coli* purine nucleoside phosphorylase (PNP), was inhibited by selected pyrazolotriazines at concentrations of 30–500 μ M. The strongest inhibitor was the 5-methylsulfanyl derivative **1**, with an IC₅₀ of ~40 μ M. Compounds methylated on the N-1 pyrazole ring nitrogen exhibited much weaker inhibitory activity, resembling that reported for the analogous pyrazolopyrimidines [17].

Plots of 1/v vs. inhibitor concentration (not shown), obtained at constant substrate concentrations, were in some instances apparently nonlinear, suggesting complex mode(s) of inhibition and/or cooperative effects [18]. The corresponding IC₅₀ values, given in Table 2, are virtually insensitive to concentration of the m⁷Guo substrate, indicating noncompetitive type of inhibition. This behaviour is not exceptional among enzymes of oligomeric structure, like bacterial PNP's [18].

Calf spleen PNP, examined under identical conditions, was not inhibited by the pyrazolotriazines. This result is not surprising since the bovine enzyme is known to exhibit much higher specificity toward purines and purine moieties of nucleosides than the *E. coli* enzyme [19].

Since the analogous pyrazolopyrimidines, e.g. allopurinol, have therapeutic applications as known strong inhibitors of the xanthine oxidase (Xox) enzyme, [20], we examined the inhibitory activities of some of pyrazolotriazines toward commercially available Xox from buttermilk. Only weak inhibition was detected in the case of the unsubstituted compound **7**, but somewhat surprisingly, moderate inhibitory activity was detected for the 5-thiomethyl derivative **1**. The title compound was apparently not a substrate for Xox, at least at the moderate enzyme concentrations employed in this work.

Table 2. Inhibition of bacterial (*E. coli*) purine-nucleoside phosphorylase and xanthine oxidase from buttermilk by the investigated pyrazolotrazines.

Compound	Inhibition of the <i>E. coli</i> PNP			Inhibition of Xox		
	substrate	concentr. [μM]	IC ₅₀ [μM]	substrate	concentr. [μM]	IC ₅₀ [μM]
7	m ⁷ Guo	32	~700	Hx	48	~800
		131	~700			
4	m ⁷ Guo	8.8	~900	Hx	145	NI ^a
		65.7	>1000			
1	m ⁷ Guo	32	~40	Hx	145	~300
		131	~30			
8	-	-	nd ^b	Hx	48	NI
9	m ⁷ Guo	32	NI	-	-	nd
		131	NI			

^aNI – no inhibition detected with inhibitor concentrations up to 300 μM.^bnd – not determined

2-Amino substituted pyrazolotriazine **4**, which may be regarded as an analogue of guanine, was examined against a rabbit muscle guanine deaminase (GDA), but did not show any activity either as a substrate, nor as an inhibitor, at least under the conditions applied in this experiment (concentrations up to 150 μM, 50 mM phosphate pH 6.1, 25°C).

Conclusions

Pyrazolotriazines are moderate inhibitors of bacterial (*E. coli*) PNP, an enzyme employed recently in cancer-oriented gene therapy experiments [21]. There is also a detectable inhibition of xanthine oxidase by the parent 3-methyl-7-azapyrazolo[4,3-d]pyrimidine, as well as its S-methyl derivative. These compounds are unique among purine analogues as having UV absorption spectra extending into the visible region (ca. 450 nm), some of them being also weakly fluorescent in aqueous solution (cf. Table 1), and thus are potentially applicable as spectroscopic probes for enzymes of purine metabolism.

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Experimental

General

All melting points are uncorrected and were determined using a Boetius melting point apparatus. Nuclear magnetic resonance (^1H -NMR) spectra were recorded on a Varian Gemini 200 MHz spectrometer in a suitable deuterated solvent using TMS as internal standard. Mass spectra were obtained on AMD 604 [electron impact (EI)] and API 350 [electrospray ionization (ESI)] spectrometers. IR spectra were measured with a Magna IR-760 spectrophotometer, and UV on a Cary 319. Fluorescence spectra were recorded using a Perkin-Elmer LS-50B spectrofluorometer, equipped with a pulsed xenon light source. *Enzymes*: xanthine oxidase (Xox) from buttermilk and purine nucleoside phosphorylase (PNP) from calf spleen were purchased from Sigma, guanine deaminase (GDA) was from ICN, and the *E. coli* PNP was a gift from Dr. W. Koszalka. Hypoxanthine and 7-methylguanosine (m^7Guo) were from Sigma. All other reagents and chemicals were obtained from Aldrich Chemical Company and were used as received, unless otherwise noted. Enzymatic assays and UV spectra were run using a Cary-319 UV spectrophotometer (Varian), equipped with a thermostatic unit. Unless otherwise indicated, all the reactions were carried out in 50 mM phosphate, pH 7.0, at 25°C. Inhibitor concentrations were evaluated spectrophotometrically, using data from Table 1. It was possible to run assays with inhibitor concentrations up to ~300 μM . Activity of PNP was assayed by following phosphorolysis of m^7Guo in 50 mM phosphate, pH 7.0 [16], the reaction monitored spectrophotometrically at 260 nm. Typical substrate concentration was ca. 9 - 135 μM . Xox activity was measured using the hypoxanthine oxidation test, with substrate concentrations 48 – 150 μM [22], monitored spectrophotometrically at 300 nm. Activity of GDA was assayed at pH 6.1, using ~50 μM 8-azaguanine as a substrate, the reaction monitored at 265 nm.

Syntheses

3-Methyl-5-methylsulfanyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazine (**1**) was obtained according to the published procedure. [12] Details of the preparation of 1,3-dimethyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazine (**8**) and 3-(2-hydroxyethoxymethyl)-1-methyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazine (**9**) will be published elsewhere [23].

1-(1-Ethoxyethyl)-3-methyl-5-methylsulfanyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazine (**2**)

To a solution of **1** (1.81g, 10 mmol) in benzene (140 mL), ethyl vinyl ether (2 mL, 20 mmol) and one drop of 38% of HCl were added. The reaction mixture was stirred at 40 °C for 8 hours, at which time a saturated solution of NaHCO_3 was added. The benzene layer was separated, dried over anhydrous MgSO_4 and concentrated *in vacuo*. The product, compound **2**, was purified by column chromatography using silica gel and chloroform as eluent. Yield 90 % (2.27 g, 9 mmol); m.p. 51 °C; ^1H -NMR (CDCl_3) δ : 1.14 (t, 3H, $J=7.0$ Hz), 1.92 (d, 3H, $J=6.0$ Hz), 2.65 (s, 3H), 2.74 (s, 3H), 3.19-3.34 (m, 1H), 3.49-3.64 (m, 1H), 6.30 (q, 1H, $J=6.0$ Hz); HRMS (ESI) for $\text{C}_{10}\text{H}_{15}\text{N}_5\text{OSNa}$ [M^+Na]: Calcd 276.0890; Found 276.0910.

1-(1-Ethoxyethyl)-3-methyl-5-methylsulfanyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazine (**3**)

To a solution of **2** (253 mg, 1 mmol) in benzene (20 mL) water (30 mL), potassium manganate(VII) (474 mg, 3 mmol), catalytic amounts of tetrabutylammonium bromide (65 mg, 0.2 mmol) and acetic acid (1.5 mL) were added. The reaction mixture was stirred at rt for 1 h. A saturated solution of Na₂S₂O₅ in water was then added to the mixture until the purple color disappeared. The organic layer was separated and the aqueous phase was extracted with benzene (3x10 mL). The combined organic extracts were dried over anhydrous MgSO₄ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (eluent: chloroform) to afford 270 mg (95%) of **3** as a yellowish oil. ¹H-NMR (CDCl₃) δ: 1.17 (t, 3H, *J*=7.0 Hz), 1.98 (d, 3H, *J*=6.0 Hz), 2.79 (s, 3H), 3.20-3.35 (m, 1H), 3.52-3.68 (m, 1H), 3.58 (s, 3H), 6.43 (q, 1H, *J*=6.0 Hz); IR (KBr) cm⁻¹: 2980, 1330, 1140; HRMS (ESI) for C₁₀H₁₅N₅O₃Na [M⁺Na]: Calcd 308.0788; Found 308.0780.

5-Amino-3-methyl-1H-pyrazolo[4,3-*e*][1,2,4]triazine (**4**)

Compound **3** (206 mg, 1 mmol) was dissolved in dry liquid ammonia (15 mL) with exclusion of moisture. The mixture was stirred at -33 °C for 1 h. After removal of the ammonia a mixture consisting of methanol (10 mL) and concentrated HCl (0.5 mL) was added. The resulting solution was stirred at rt for 12 h, then the solvent was removed and the crude product was purified by column chromatography (silica gel, eluent: chloroform-ethanol, 20:1) to give compound **7** in 80% yield. This compound slowly decomposed at approximately 256 °C. ¹H-NMR (DMSO) δ: 2.38 (s, 3H), 6.97 (s, 2H), 11.05 (broad, pyrazole NH); IR (KBr) cm⁻¹: 3340, 2850, 1650; HRMS (EI) for C₅H₆N₆ [M⁺]: Calcd 150.06539; Found 150.06582.

1-(1-Ethoxyethyl)-3-methyl-5-hydrazino-1H-pyrazolo[4,3-*e*][1,2,4]triazine (**5**)

To the solution of **3** (285 mg, 1 mmol) in dry THF (10 mL) cooled to 0-5 °C, anhydrous hydrazine (0.1 mL, 3 mmol) was added. The reaction was stirred at 0-5 °C for 30 min and an additional 5 h at rt. After that time the solvent was evaporated *in vacuo* and the crude product was recrystallized from ethanol to give 213 mg (90%) of **5** as an orange solid. M.p. 120-123 °C; ¹H-NMR (CDCl₃) δ: 1.14 (t, 3H, *J*=7.0 Hz), 1.91 (d, 3H, *J*=6.0 Hz), 2.55 (s, 3H), 3.21-3.36 (m, 1H), 3.48-3.63 (m, 1H), 6.22 (q, 1H, *J*=6.0 Hz), 6.84 (s, 1H); HRMS (ESI) for C₉H₁₅N₇ONa [M⁺Na]: Calcd 260.1230; Found 260.1226.

1-(1-Ethoxyethyl)-3-methyl-1H-pyrazolo[4,3-*e*][1,2,4]triazine (**6**)

To the solution of compound **4** (237 mg, 1 mmol) in dry ethanol (20 mL) yellow mercury oxide(II) (1.08 g, 5 mmol) was added and the mixture was heated at 40 °C for 0.5 h. After that time the reaction mixture was filtered off and the solvent was evaporated *in vacuo*. The crude product was purified by column chromatography (silica gel, eluent: chloroform) to give 124 mg (60 %) of **7** as a yellow oil. ¹H-NMR (CDCl₃) δ: 1.14 (t, 3H, *J*=7.0 Hz), 1.93 (d, 3H, *J*=6.0 Hz), 2.72 (s, 3H), 3.19-3.34 (m, 1H), 3.51-3.66 (m, 1H), 6.38 (q, 1H, *J*=6.0 Hz), 9.76 (s, 1H); IR (KBr) cm⁻¹: 2980, 1130; HRMS (ESI) for C₉H₁₃N₅ONa [M⁺Na]: Calcd 230.1012; Found 230.1030.

3-Methyl-1H-pyrazolo[4,3-*e*][1,2,4]triazine (**7**)

A mixture of **5** (206 mg, 1 mmol) and concentrated HCl (0.5 mL) in methanol (10 mL) was stirred at room temperature for 12 h. Then the solvent was removed, and the residue was separated by column chromatography (silica gel, eluent: chloroform-ethanol, 20:1) to afford 124 mg (92 %) of **6** as a yellow solid. M.p. 159-161 °C; ¹H-NMR (CDCl₃) δ: 2.76 (s, 3H), 9.82 (s, 1H), 11.10 (broad, pyrazole-NH); HRMS (EI) for C₅H₅N₅[M⁺]: Calcd 135.05450; Found 135.05403.

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Sample Availability: Available from the authors.