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2-Alkyloxyalkylthiohypoxanthines as new potent inhibitors of xanthine oxidase

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Abstract

The title compounds were prepared and tested as xanthine oxidase (XO) inhibitors. Results evidenced that potency was related to the position of the oxygen atom in the 2-linear chain and that it grew with distance from the sulfur atom until it became equipotent to 2-*n*-hexylthiohypoxanthine. Enzymatic oxidation on C(2) occurred in the 8-alkylthiohypoxanthines. On the contrary, oxidation on C(8) did not occur in the 2-alkythioderivatives, demonstrating that the chain forced these molecules to form a complex with molybdenum(VI) involving only the N(3) and N(9) nitrogen atoms.

Keywords: Xanthine oxidase; Inhibitors; 2-Thiohypoxanthine; 2-Mercaptohypoxanthine; 2-Thioxo-1,2,3,7(9)-tetrahydro-purin-6-one

1. Introduction

The enzyme xanthine oxidase (XO) catalyses the hydroxylation of hypoxanthine and of xanthine to yield uric acid and superoxide anions. These superoxide anions have been linked to postischaemic tissue injury and oedema [1] as well as to vascular permeability [2]. XO can also oxidise synthetic purine drugs, such as antileukaemic 6-mercaptopurine, with loss of their pharmacological properties [3]. Then, the control of the action of XO may help the therapy of some diseases. Today, the therapy of gout makes use of allopurinol, a potent inhibitor of XO known for a long time [4]. However, given its side effects and its inability to prevent the formation of free radicals by the enzyme [5], the research on new XO inhibitors is in progress.

In the past we reported the good activity as inhibitors of XO of 2-alkylhypoxanthines and 2-alkyl-8-azahypoxanthines [6–10] and a type-II binding with enzyme was hypothesised according to the scheme proposed by Robins et al. [4]. We demonstrated that near the active site of the enzyme only one lipophilic narrow pocket, characterised by well-defined dimensions, existed which was able to receive a linear alkyl chain; this goal was obtained through the same favourable interaction with both 2-*n*-alkylhypoxanthines and 8-*n*-alkylhypoxanthines [6–8]. In the first case, 2-substituted compounds demonstrated good inhibitory activity whereas, in the second case, 8-substituted derivatives showed substrate behaviour. Further, the fact that 2-*n*-alkoxycarbonyl-8-azahypoxanthines were more active than the corresponding 2-*n*-alkyl-8-azahypoxanthines [9] allowed us to hypothesise that the part of this pocket nearest to the site of interaction of the enzyme with the purine nucleus has some residues capable of polar interactions with atoms, bearing electron lone pairs, at the beginning of the chain. This concept was supported with the preparation, among others, and biological evaluation of 2-*n*-alkylthio-hypoxanthines, which were very potent. In fact, the 2-*n*-hexylthiohypoxanthine had a K_i value of 9.8 nM, thus being 700 times more potent than allopurinol [10].

Starting from this lead compound, we projected to further explore the pocket facing the C(2) position of the purine nucleus by the modification of the linear chain bound to it. In the present paper we describe the preparation and biological evaluation of the title compounds with the aim of obtaining some other information about the enzyme zone which interacts with the side-chain.

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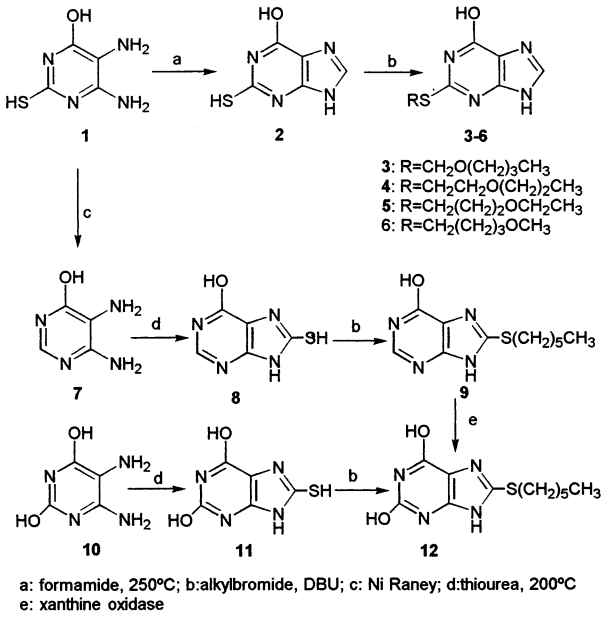


Scheme 1. Synthetic routes to compounds **3-6**, **9**, **12**.

2. Chemistry

The synthetic routes are summarised in Scheme 1. The 2-thiohypoxanthine (**2**) was obtained by cyclisation of 4,5-diamino-6-hydroxy-2-thiopyrimidine (**1**) with anhydrous formamide, as reported by Beaman in 1954 [11] at very high temperatures (200–250 °C); the very good yield obtained (94%) was higher than the one reported in the original article.

Compound **2** reacted with the suitable bromoalkylether, obtained by known methods [12–18], in the presence of 1,8-diazabicyclo-(5.4.0)-undec-7-ene (DBU) and *N*,*N*-dimethylformamide to give compounds **3**–**6**.

By fusion of 4,5-diamino-6-hydroxypyrimidine sulfate (**7**) or 4,5-diamino-2,6-dihydroxypyrimidine sulfate (**10**) and thiourea, the 8-thio-hypoxanthine (**8**) [19] and the 8-thio-xanthine (**11**) [20], respectively, were obtained in a good yield. Compound **11** was also obtained by heating 4,5-diamino-2,6-dihydroxypyrimidine sulfate with carbon disulfide in ethanol at 120 °C [20]. Compound **9** was obtained by the reaction of **8** with bromohexane, DBU and *N*,*N*-dimethylformamide [21]; compound **12** was obtained by the same chemical method starting from **11**, or by the enzymatic reaction with XO from 8-hexylthio-hypoxanthine (**9**).

3. Experimental

3.1. Chemistry

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. IR spectra in Nujol mulls were recorded on a Perkin–Elmer Model 1310 spectrometer. ^{1}H NMR spectra were recorded on a Bruker AC 200 spectrometer in δ units from TMS as an internal standard; the compounds were dissolved in the DMSO. HPLC were carried out on a Violet PM 900 with a PH-410 UV photometer as detector, and a Nucleosil C18 column (15 × 4.8 mm) using as eluent MeOH–phosphate buffer pH 7.5 70:30.

Mass spectra were performed on a Hewlett–Packard GC/MS System 5988A. TLC was performed on precoated silica gel F_{254} plates (Merck) or on precoated RP-18 F_{254} plates (Merck). Flash-column chromatographies were performed using Merck Kieselgel 60 (230–400 mesh). Microanalyses (C, H, N) were carried out on a Carlo Erba elemental analyser (Model 1106) and were within ± 0.4% of the theoretical values.

3.1.1. General synthesis of 2-alkyloxyalkylthio-hypoxanthines (3–6)

To a solution of **2** (1.68 g, 10 mmol) in the minimum amount of *N*,*N*-dimethylformamide, DBU (1.52 g, 10 mmol) and the suitable bromoalkylether (Table 1) (1.66 g, 10 mmol) were added. The mixture was stirred for 24–52 h (Table 1) at room temperature, then water was slowly added until a solid precipitated. After filtration the solid was flash-chromatographed using as eluent $CHCl_3$–MeOH 9:1 obtaining the pure product which crystallised from MeOH (Tables 1 and 2).

Table 1
Reaction and physical data of compounds **3**–**6**, **9** and **12**

Comp.	Bromoalkylether	Reaction time (h)	Empirical formula (molecular weight)	M.p. (°C)	R_f	Yield (%)
3	$BrCH_2O(CH_2)_3CH_3$	24	$C_{10}H_{14}N_4O_2S$ (254)	275	0.28 [a]	42
4	$BrCH_2CH_2O(CH_2)_2CH_3$	48	$C_{10}H_{14}N_4O_2S$ (254)	280	0.27 [a]	74
5	$BrCH_2(CH_2)_2OCH_2CH_3$	48	$C_{10}H_{14}N_4O_2S$ (254)	277	0.30 [a]	48
6	$BrCH_2(CH_2)_3OCH_3$	52	$C_{10}H_{14}N_4O_2S$ (254)	265	0.25 [a]	35
9	$Br(CH_2)_5CH_3$	12	$C_{11}H_{16}N_4OS$ (252)	268	0.37 [b]	56
12	$Br(CH_2)_5CH_3$	48	$C_{11}H_{16}N_4O_2S$ (268)	268	0.31 [b]	53

[a] Silica gel, eluent $CHCl_3$–MeOH 9:1.
[b] RP18, eluent MeOH–H_2O 6:4.

Table 2
Spectroscopic data for compounds **3**–**6**, **9**, and **12**

Comp.	^{1}H NMR			MS
	Aromatic H	Aliphatic H	Exchang. H	*m/z* (%)
3	8.13 (s, 1H)	5.48 (s, 2H); 3.59 (m, 2H); 1.45 (m, 2H); 1.24 (m, 2H); 0.82 (t, 3H)		254 (M^+, 17.3); 168 (21); 57 (100)
4	7.99 (s, 1H)	3.62 (s, 2H); 3.62 (m, 2H); 3.36 (m, 4H); 1.49 (m, 2H); 0.84 (t, 3H)	8.31 (1H)	254 (M^+, 6.8); 168 (100); 110 (23); 43 (38)
5	8.08 (s, 1H)	4.18 (s, 2H); 3.28 (m, 2H); 3.10 (m, 2H); 1.95 (m, 2H); 1.1 (t, 3H)	12.2 (1H)	254 (M^+, 1.1); 208 (65); 168 (100); 44 (97)
6	7.61 (s, 1H)	3.37 (m, 7H); 2.03 (m, 4H)		254 (M^+, 5.1); 168 (100); 110 (43)
9	7.89 (s, 1H)	3.18 (t, 2H); 1.62 (m, 2H); 1.22 (m, 6H); 0.81 (m, 3H)	12.2 (1H)	252 (M^+, 5.5); 205 (27); 168 (100); 43 (29)
12	7.92 (s, 1H)	3.12 (t, 2H); 1.58 (t, 2H); 1.24 (m, 6H); 0.82 (t, 3H)	11.3 (2H)	268 (M^+, 15.6); 184 (100); 85 (58); 43 (42)

*3.1.2. 8-Hexylthio-hypoxanthine (**9**)*

To a solution of 8-thio-hypoxanthine (**8**) (0.20 g, 1.19 mmol) in the minimum amount of *N*,*N*-dimethylformamide, DBU (0.18 g, 1.18 mmol) and 1-bromohexane (0.19 g, 1.18 mmol) were added. The mixture was stirred for 12 h at room temperature, then water was slowly added until a solid precipitated. After filtration, the solid was crystallised from MeOH to give pure **9** (0.168 g, 0.66 mmol) (Tables 1 and 2).

*3.1.3. 8-Hexylthio-xanthine (**12**)*

To a solution of 8-thioxanthine (**11**) (0.50 g, 2.72 mmol) in the minimum amount of *N*,*N*-dimethylformamide, DBU (0.41 g, 2.69 mmol) and 1-bromohexane (0.41 g, 2.51 mmol) were added. The mixture was stirred for 48 h at room temperature, then water was slowly added until a solid precipitated. After filtration, the solid was flash-chromatographed using as eluent $CHCl_3$–MeOH 9:1 obtaining pure product which crystallised from MeOH to give **12** (0.386 g, 1.44 mmol) (Tables 1 and 2). Compound **12** was obtained also by the following enzymatic method. To a solution of **9** (10 mg, 0.04 mmol) in 1 ml of DMSO and 80 ml of phosphate buffer at pH 7.6 was added XO (see Section 3.2) (40 μl); the mixture was stirred and air bubbled at 35 °C for 12 h, then was evaporated and the residue was analysed by HPLC showing only a peak at the same retention time as compound **12** obtained by chemical reaction.

3.2. Biochemistry

XO (from buttermilk, 1.36 U/mg) was purchased from Boehringer (Boehringer Mannheim Italia S.p.A.) and hypoxanthine from Fluka (Sigma–Aldrich s.r.l.) and used as a 100 μM solution in a Tris buffer–HCl at pH 7.6. Compound **9** was used as substrate in 100 μM DMSO solution. XO activity was assayed spectrophotometrically in an air-saturated Tris buffer–HCl at pH 7.6, $I = 0.1$ at 25 ± 0.2 °C using a Beckman DU 50 spectrophotometer with a thermostated cuvette holder. The increase in uric acid (enzymatic reaction product) concentration was evaluated at 295 nm with hypoxanthine as substrate (10 μM) and XO sufficient to obtain an average reaction rate for the control reaction of 0.100 ± 0.005 absorbance units/min. The inhibitors in question were dissolved in DMSO. DMSO concentration in all the assays was kept at 3% v/v, which has no effect on XO activity. IC_{50} values (the concentration required to produce 50% inhibition of the enzyme catalysed reaction) were determined from least-square analysis of the linear portion of log dose–inhibition curves. Each curve was generated using at least three concentrations of inhibitor producing an inhibition between 20 and 80%, with three replicates at each concentration. K_M determination for compound **9** was performed by plotting 1/V versus 1/[S] (2–6 μM) reading the absorbance change at 300 nm which evaluated the increase in **12** (enzymatic reaction product) concentration.

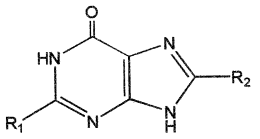
Kinetic studies were performed with at least three different concentrations of inhibitor in the presence of variable concentrations of hypoxanthine (6–10 μM). K_i values (the dissociation constants of the enzyme–inhibitor complex) were determined from the slopes in double reciprocal plots [22].

4. Results and discussion

Biological results showed that the introduction of an oxygen atom in the linear chain leads to a modulation of the inhibitory potency among tested compounds (Table 3). In particular, IC_{50} values decreased little by little with the increase of oxygen atom distance from the sulfur atom, i.e. **6** (IC_{50} 28 nM) < **5** (IC_{50} 58 nM) <

4 (IC_{50} 670 nM) < **3** (IC_{50} 20 560 nM), allopurinol IC_{50} 4200 nM. Compound **6**, K_i 19 nM, resulted almost equipotent to compound **13**, K_i 9.8 nM, prepared and assayed in a previous paper [10], having a linear thioalkyl chain in 2 position. The trend registered among compounds **3**, **4**, **5**, and **6** could be imputed at the start to a different conformation of the chain within the lipophilic pocket with respect to the usual description of the linear chains, especially in the case of compound **3**. In this case, in which a methylene unit was interposed between the sulfur and oxygen atoms, lone pairs of these two atoms, by their reciprocal repulsive action, should force, in our opinion, the linear chain to assume a conformation different from the linear one. Instead, molecular modelling with minimisation of energy (Chemoffice and Insight II programs) indicated that the chain retained a linear conformation in all cases as in the thioalkyl one. Therefore, the low potency of compound **3** could be explained taking into account the repulsive effect provoked by a negative charged group facing the oxygen atom of the chain. This group could be the negative part of a protein fragment with dipole characteristics, whose positive part, near the narrow entrance of the lipophilic pocket, influenced positively the bind with the sulfur atom. Then as the S–O distance grew from **3** to **6** the repulsive effect became progressively smaller and smaller.

Table 3
Biological results



R_1 R_2

Comp.	R_1	R_2	IC_{50} (nM)	K_i or K_m (nM)
3	$SCH_2O(CH_2)_3CH_3$	H	20 560	
4	$SCH_2CH_2O(CH_2)_2CH_3$	H	670	
5	$SCH_2(CH_2)_2OCH_2CH_3$	H	58	K_i: 23
6	$SCH_2(CH_2)_3OCH_3$	H	28	K_i: 19
9	H	$S(CH_2)_5CH_3$	560	K_m: 3860
12	OH	$S(CH_2)_5CH_3$	660	
13	$S(CH_2)_5CH_3$	H		K_i: 9.8 [a]
Allopurinol			4200	K_i: 7000 [b]

[a] See Ref. [10].
[b] See Ref. [4].

Furthermore, to clarify the binding mechanism of 2-alkyloxy-alkylthiohypoxanthines with active site of XO, we prepared 8-hexylthio-hypoxanthine (**9**) and its 2-hydroxy analogue (**12**).

Both these compounds showed an inhibitory behaviour with IC_{50} 560 and 660 nM, respectively. Compound **9**, contrary to the title compounds, resulted also a substrate as it was transformed by enzyme (XO) oxidation in a compound which resulted identical to compound **12** by HPLC analysis. These results indicated that the enzyme active site was able to accept both compounds with the linear chain in position 2 or 8. However the enzyme was able to oxidise in position 2 only the compounds bearing a chain on C(8) like **9** (Fig. 1), whereas oxidation on C(8) in compound like **6**, having the chain in position C(2), was not allowed probably owing to too long a distance between the sulfur anion of the enzyme prosthetic group and C(8) (Fig. 2).

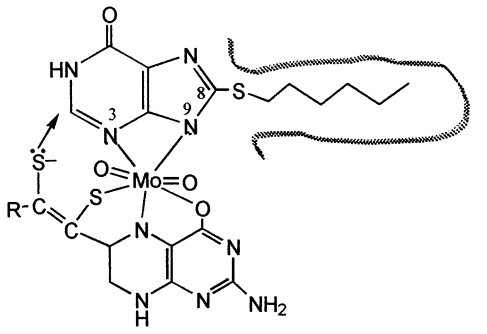


Fig. 1. Type II binding of C(8) substituted compounds: oxidation on C(2) is allowed.

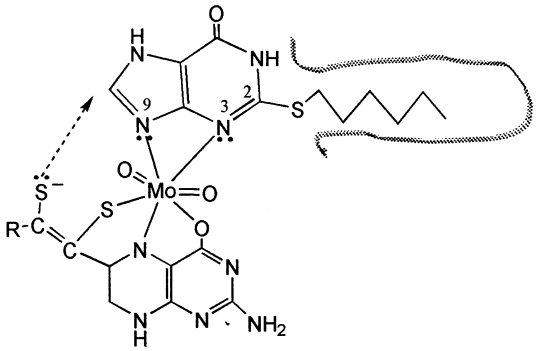


Fig. 2. Type II binding for C(2) substituted compounds: oxidation on C(8) is not allowed.

This last result can be explained hypothesising that only N(3) and N(9) atoms, according to the binding type II proposed by Robins [4], participated in the complex with molybdenum(VI) present in the enzyme active site; molecules like **9** or **6** would orient themselves within it in a way suitable to insert the linear chain inside the lipophilic pocket (Figs. 1 and 2). Oxidation on C(8) in compounds with the chain in position 2 did not occur since the chain position did not allow the formation of the complex of molybdenum(VI) with O^6 and N(7) according to Robins binding type I (Fig. 3).

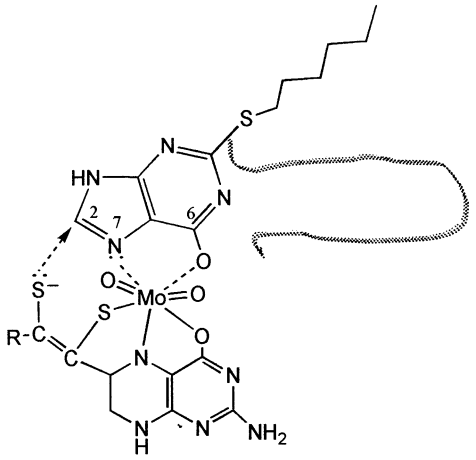


Fig. 3. Type I binding is not allowed for C(2) substituted compounds.

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