Synthesis of 3-Alkyl-6-Phenyl-4(3*H*)-pteridinones and their 8-Oxides. Potential Substrates of Xanthine Oxidase

J. W. G. De Meester, W. Kraus [1], and H. C. van der Plas*

Department of Organic Chemistry, Wageningen Agricultural University, De Dreyen 5, 6703 BC Wageningen, The Netherlands

H. J. Brons and W. J. Middelhoven

Department of Microbiology, Wageningen Agricultural University, Hesselink van Suchtelenweg 4 6703 CT Wageningen, The Netherlands

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Synthetic routes for the preparation of 3-alkyl-6-phenyl-4(3*H*)-pteridinones **6** and their corresponding 8-oxides **5** (R = CH_3, C_2H_5, $(CH_2)_2CH_3$, $(CH_2)_3CH_3$, $CH(CH_3)C_2H_5$, $CH(CH_3)_2$ and $CH(C_2H_5)CH_2OCH(OC_2H_5)_2$ are described and their reactivities towards xanthine oxidase from *Arthrobacter* M-4 are determined. Only the 3-methyl derivative of 6-phenyl-4(3*H*)-pteridinone and its 8-oxide *i.e.* **6a** and **5a** are found to be substrates although their reactivities are still very low. Oxidation takes place at C-2 of the pteridinone nucleus. All the 3-alkyl derivatives are less tightly bound to the enzyme than 6-phenyl-4(3*H*)-pteridinone. Introduction of the *N*-oxide at N-8 considerably lowers the binding of the substrates. Inhibition studies have revealed that 3-methyl-6-phenyl-4(3*H*)-pteridinone (**6a**) is a non-competitive inhibitor with a Ki-value of 47 μM and the 3-ethyl derivative (**6b**) an uncompetitive one with a Ki-value of 19.6 μM.

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For several years there is current interest in our laboratory in the behaviour of 4(3*H*)-pteridinones particularly the 6- and 7-aryl derivatives, towards xanthine oxidase [2-5]. Earlier investigations have shown that 7-aryl-(3*H*)-pteridinones are more easily oxidized into the corresponding 7-aryl-2,4(1*H*,3*H*)-pteridinediones (lumazines) [2a,2c,3] by milk xanthine oxidase (MXO) than the 7-alkyl-4(3*H*)-pteridinones [2b,2c]. With *Arthrobacter* M-4 xanthine oxidase (AXO) 6-aryl-4(3*H*)-pteridinones [2d,3,4] were also exclusively oxidized at C-2. These results strongly support the idea that hydrophobic interaction between the phenyl group present in the pteridinone and hydrophobic group(s) in the active site of the enzyme is of importance in the formation of the enzyme-substrate complex, thus strongly influencing the rate of the reaction. This interaction has also been put forward as a possible explanation of the large inhibitory capacity found for 6-aryl-4(3*H*)-pteridinones in MXO mediated reacttions [5]. Similar results are also observed with the 8- and 9-phenylpurines and their analogs [6].

The rate of the enzymatic reactions of the *N*-methyl derivatives of hypoxanthine and xanthine by mammalian [7a,7c,7d,7e] and bacterial [7b,7f,7g] xanthine oxidases has been reported to be strongly affected by both the position and number of methyl groups [7]. Similarly *N*-methylation of 4(3*H*)-pteridinone derivatives may increase or reduce rates of enzymatic oxidation [8]. Since little is known about the influence of 3-alkyl groups in 6-aryl-4(3*H*)-pteridinones on the rate of the oxidation with xanthine oxidase, we prepared a series of 3-alkyl-6-phenyl-4(3*H*)-pteridinones **6** and studied the reactivity of each of them towards xanthine oxidase from *Arthrobacter* M-4. Since purine 1-oxides are converted into 2-hydroxypurines by MXO [9] and AXO is able to convert 6-phenyl-4(3*H*)-pteridinone 8-oxide into the corresponding lumazine 8-oxide [3], we included also the 3-alkyl-6-phenyl-4(3*H*)-pteridinone 8-oxides **5** in our study.

In this paper the syntheses of the compounds **5** and **6** are described, spectroscopic evidence is presented supporting the structures assigned and results of the reaction of **5** and **6** with AXO are discussed.

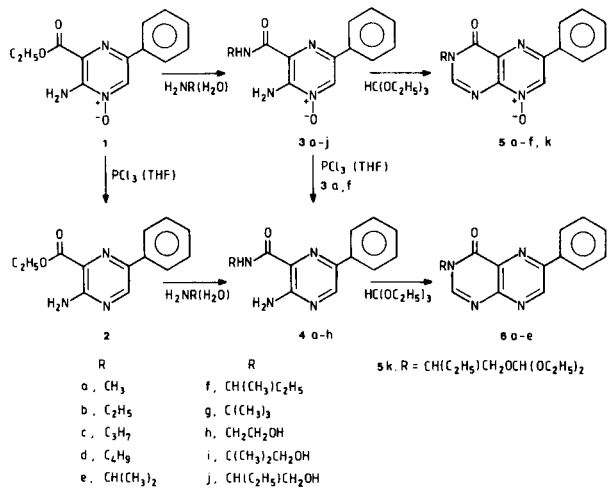
Syntheses.

The key intermediate in our syntheses (Scheme 1) is 2-amino-3-ethoxycarbonyl-5-phenylpyrazine 1-oxide (**1**) [10]. The ethoxycarbonyl group in **1** reacts readily with amines to give the corresponding amides **3**. As amines we used methyl-, ethyl-, *n*-propyl-, *n*-butyl-, *i*-propyl-, *s*-butyl, *t*-butyl-, 2-hydroxyethyl-, (1-hydroxybutyl-2-)- and (1-hydroxy-2-methylpropyl-2-)-amine. For the preparation of **3a-d** pure amines could be used, while for **3h-j** a 40% aqueous solution was preferred. With *t*-butylamine addition of water was found to be necessary to complete the reaction.

The cyclization of the amides **3** into 3-alkyl-6-phenyl-4(3*H*)-pteridinone 8-oxides **5** was performed by moderate heating with triethyl orthoformate. The best results for cyclization were obtained by heating the purified amides **3** with triethyl orthoformate in an open flask at 145°. The ring closure works satisfactorily with primary amides **3a-d** but gave distinctly lower yields with the secondary amides **3e, 3f, 3j**. With the tertiary amides **3g, 3i** no cyclization occurs. An analogous influence of the alkyl group on the cyclization behaviour has been reported in the ring closure of *o*-aminobenzoic alkylamides into 3-alkyl-4(3*H*)-quinazolones [11]. When the amides **3** are pure, the compounds **5** precipitate after cooling of the reaction mixture;

the materials obtained, after washing with ethanol and ether, are then analytically pure. Addition of a solvent like dimethylformamide [10] gave less satisfactory results.

Scheme 1

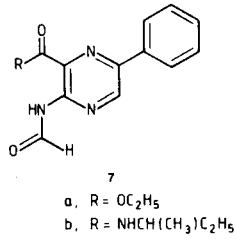


It was observed that ring closure of the amide **3j**, containing on N-3 an α-hydroxy group led to the formation of compound **5k**, containing the orthoester of formic acid in the side chain.

Removal of the *N*-oxide function from pteridine 8-oxides can be achieved by reduction with an aqueous sodium dithionite solution followed by treatment with potassium permanganate [4,10]. We did not use this procedure to prepare **6** but investigated an alternative route, *i.e.* cyclization of 2-amino-3-alkylcarbamoyl-5-phenylpyrazines **4** by treatment with triethyl orthoformate according to the procedure as described above for **3** into **5**. It was found that the best method to obtain **4** is not reduction of **3** with phosphorus trichloride [4,12] in tetrahydrofuran [13] but first reduction of **1** into 2-amino-3-ethoxycarbonyl-5-phenylpyrazine (**2**) (yield about 80%) and then replacement of the ethoxy group in **2** by an alkylamino group. This alkylamino-deethoxylation reaction occurs readily with primary amines. Addition of water was again necessary to complete the reaction with secondary and tertiary amines (for example no reaction occurred if *t*-butylamine was heated with **2** for three hours at 120°, but in the presence of water a yield of 92% was obtained).

Whereas for the ring closure a mixture of triethyl orthoformate and acetic anhydride is usually applied [4,14b], we observed that the cyclization of **4a-e** into **6a-e** by triethyl orthoformate only (thus without acetic anhydride) takes place in yields far superior to those obtained in the presence of acetic anhydride; it led to the desired compounds in pure form. In the presence of acetic anhydride a dark brown mixture is formed. Since in the case of pyrazine 1-oxides, addition of acetic anhydride to the reaction might lead to rearrangements [12] experiments were conducted in the presence of the last mentioned reagent.

Scheme 2



Ring closure of **4f** into **6f** with triethyl orthoformate failed; from the reaction mixture only the 2-formylaminopyrazine **7b** [15] could be isolated as the sole product in about 3% yield. A similar 2-formylamino compound **7a** was obtained on reaction of **2** with triethyl orthoformate and acetic anhydride. The structures of the compounds **7** were assigned by ^{1}H nmr spectra since attempts to purify them led to hydrolysis of the formyl group; **2** and **4f** were formed from **7a** and **7b** respectively.

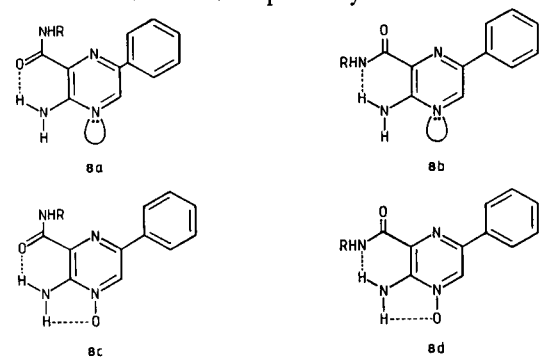


Figure 1. Possible configurations for compounds **3** and **4**: **8a** and **8b** are the conformations for **3**, while **8c** and **8d** are assumed to be the conformations for **4**.

Initial ^{1}H-nmr studies [16] indicate that compounds **3-4** adopt the s-*trans* configuration involving the C(3)=N bond and the carbonyl function of the amide as depicted in Figure 1 (**8a, 8c**). In compounds **3** the 2-amino group is involved in an intramolecular H-bond with the *N*-oxide function. The forced conditions being used to achieve cyclization is apparently due to the absolute necessity of alteration of the conformations from s-*trans* **8a** and **8c** assumed currently for **3** and **4** to s-*cis* **8b** and **8d** which allowed

Comparison of the chemical shifts of the ring protons H-6 in the compounds **3** and **4** (see Table 1) reveals that in the 1-oxides **3** H-6 is substantially deshielded (0.22 - 0.27 ppm). A similar deshielding was observed for the protons of the amino group in position 2 (0.25-0.28 ppm). This effect has also been observed for H-6 in **1** compared to that in **2**. This deshielding effect of H-6 is unexpected since it

Table 1

^{1}H NMR Spectral Data of 2-Amino-3-(alkylcarbamoyl)-5-phenylpyrazines **4** and their 1-Oxides **3** (δ-values) [a]

Alkyl substituent	Compound	H-6	N-CH [c]	Compound	H-6	N-CH [c]	Δ H6 [b]
CH_3	**3a**	9.10 (s)	2.87 (3H, d)	**4a**	8.88 (s)	2.86 (3H, d)	0.22
CH_2CH_3	**3b**	9.10 (s)	3.35 (2H, q)	**4b**	8.85 (s)	3.40 (2H, q)	0.25
$CH_2CH_2CH_3$	**3c**	9.10 (s)	3.30 (2H, q)	**4c**	8.85 (s)	3.30 (2H, q)	0.25
$(CH_2)_3CH_3$	**3d**	9.10 (s)	3.30 (2H, q)	**4d**	8.87 (s)	3.30 (2H, q)	0.23
$CH(CH_3)_2$	**3e**	9.07 (s)	4.16 (1H, m)	**4e**	8.83 (s)	4.16 (1H, m)	0.24
$CH(CH_3)C_2H_5$	**3f**	9.07 (s)	3.97 (1H, m)	**4f**	8.85 (s)	3.95 (1H, m)	0.22
$C(CH_3)_3$	**3g**	8.80 (s)	—	**4g**	8.53 (s)	—	0.27
CH_2CH_2OH	**3g**	9.10 (s)	3.48 (4H, m)	**4h**	8.83 (s)	3.50 (4H, m)	0.27
$C(CH_3)_2CH_2OH$	**3i**	9.08 (s)	—				
$CH(C_2H_5)CH_2OH$	**3j**	9.08 (s)	3.93 (1H, m)				

[a] Resonance signals of the 5-phenyl ring protons are over a range of 8.03-8.23 ppm (2H) and 7.42-7.48 ppm (3H) for **3** and **4**; 2-amino protons are over a range of 7.88-7.93 ppm for **3** and 7.63-7.65 ppm for **4**, whereas the 3-CONH protons are found over a range of 8.85-8.99 ppm for **3a-d,h** and 8.35-8.52 ppm for **4e,f,i,j** respectively. [b] Δ H-6 = δ H-6 (**3**) - δ H-6 (**4**). [c] Resonance signals of the α-protons of 3-alkyl substituents.

Table 2

^{1}H NMR Spectral Data of 3-Alkyl-6-phenyl-4(3*H*)-pteridinones **6** and their 8-Oxides **5** (δ-values [a]

Alkyl substituent	Compound	H7	N-CH [b]	Compound	H7	N-CH [b]	Δ H7 [c]
CH_3	**5a**	9.38 (s)	3.55 (3H, s)	**6a**	9.58 (s)	3.58 (3H, s)	−0.20
CH_2CH_3	**5b**	9.38 (s)	4.03 (2H, q)	**6b**	9.60 (s)	4.08 (2H, q)	−0.22
$CH_2CH_2CH_3$	**5c**	9.38 (s)	4.00 (2H, t)	**6c**	9.62 (s)	4.03 (2H, t)	−0.24
$(CH_2)_3CH_3$	**5d**	9.38 (s)	4.03 (2H, t)	**6d**	9.62 (s)	4.06 (2H, t)	−0.24
$CH(C_3)_2$	**5e**	9.38 (s)	4.98 (1H, m)	**6e**	9.60 (s)	5.03 (1H, m)	−0.22
$CH(CH_3)C_2H_5$	**5f**	9.37 (s)	4.78 (1H, m)				
$CH(C_2H_5)CH_2OCH(OC_2H_5)_2$	**5k**	9.36 (s)	4.70 (1H, m)				

[a] Resonance signals of the 6-phenyl ring protons are over a range of 8.18-8.25 ppm (2H) and 7.46-7.57 ppm (3H). [b] Resonance signals of α-protons of 3-alkyl substituents. [c] Δ H-7 = δ H-7 (**5**) - δ H-7 (**6**).

has been shown that the *N*-oxide function causes shielding of the *ortho* protons in pyrazine ring due to anisotropic and inductive effects of the N-O linkage [17]. This deshielding effect could not be attributed to *N*-alkyl substitution of the carbamoyl function since the same effect is also observed for **1** and **2**; quite recently this effect was also found for other 5-substituted 2-amino-3-carbamoylpyrazines [4,18].

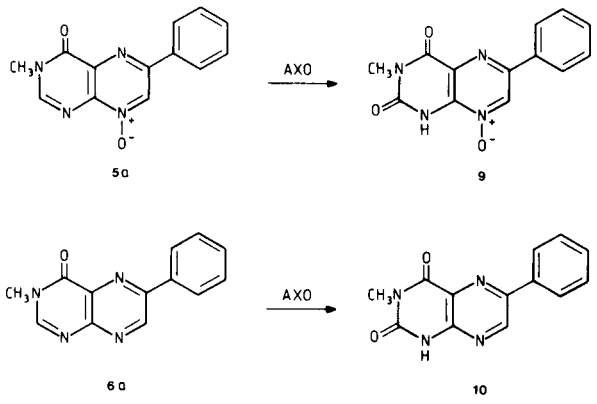
Examination of the chemical shifts of H-7 in the compounds **5** and **6** however revealed the usual shielding effect of H-7 in the 8-oxides **5** (see Table 2). Comparing the chemical shifts of the H-6 protons and the related H-7 protons within the pairs **3** and **5** as well as **4** and **6** it is evident that the ring closure results in an overall deshielding effect of about 0.3 ppm and 0.7 ppm, respectively, which parallels the differences in electron density distribution [19] in the pteridine rings of **5** and **6**.

Enzymatic Oxidation.

Since the *p*H optimum of AXO is about 7.2 [3,4,20], as was established with xanthine and 1-methylxanthine, the oxidation was carried out at this *p*H. After a 100 μ*M* solution of **6a** was incubated with AXO, a slow conversion of **6a** took place, as observed by uv-spectroscopy. After about thirty hours of incubation at 25°, no further changes in the uv-spectrum were found. The final spectrum was identical to that of 3-methyl-6-phenyllumazine (**10**) [21b,21c] indicating that oxidation in **6a** took place at C-2, just as ob-

served in the oxidation of 6-phenyl-4(3*H*)-pteridinone (**6**, R = H) [4]. At somewhat higher *p*H (7.5 and 8.0) hardly any conversion was observed, in agreement with earlier report [3,22]. The oxidation at *p*H = 7.2 was too slow for accurate determination of the kinetic parameters. However by comparison of the time for completing the oxidation, the oxidation rate of **6a** is judged to be 1% of that of 6-phenyl-4(3*H*)-pteridinone [4] (when using the same substrate concentration of 100 μM).

Scheme 3



5a 9

6a 10

Also treatment of 3-methyl-6-phenyl-4(3*H*)-pteridinone 8-oxide (**5a**) with AXO resulted in oxidation at C-2, as indicated by the formation of an absorption maximum in the incubation mixture, being the same as that of an authentic specimen of 3-methyl-6-phenyllumazine 8-oxide **9** [21b,21c,23]. However the rate of oxidation is much lower than that of **6a**, as it requires 120 hours to convert about 50% of **5a** at a concentration of 100 μM! It is evident that the presence of the methylgroup at N-3 in **5a** decreases the rate considerably and that introduction of a *N*-oxide function at position 8 further decreases the rate of oxidation. When the methyl group in **5a** and **6a** is replaced by more bulky alkylgroups or by groups containing a α-hydroxy group (see structures **5b-f,k** and **6b-e**) no conversion was observed for all of them during twelve hours of incubation. No attempts were made to oxidize these compounds with immobilized cells [2d,3,4].

Table 3

Inhibition Parameters (I_{50}- and Ki-values) for the 3-Alkyl-6-phenyl-4(3*H*)-pteridinones (**6**) at *p*H = 7.25 using 100 μM 1-Methylxanthine as Substrate [a]

	I_{50} [b]	K_i [b]
6-Phenyl-4(3*H*)-pteridinone (**6**, R = H)	6.9 ± 0.8	
6a	46 ± 8	47
6b	54 ± 9	19.6
6c	63 ± 10	
6d	70 ± 6	
6e	57 ± 7	

[a] The activity of the cell-free extracts used in this study was 0.27 μmole + 0.04 μmole/min.mg. [b] In μmole/l.

Although the rates of oxidation of the 3-alkyl-6-phenyl-4(3*H*)-pteridinones (**6**) and their 8-oxides **5** are very low, it does not exclude the possibility that these substrates are bound to the enzyme and in fact might act as inhibitors. In order to evaluate the affinity of the compounds **5** and **6** for the bacterial enzyme we estimated the I_{50}-value at *p*H = 7.25, using 1-methylxanthine as substrate (100 μM) [24]. The I_{50}-value obtained for the compounds **6a-e** are summarized in Table 3. This table clearly shows that increase of the bulkiness of the alkylgroup at position 3 in **6** results in an about seven times lower affinity towards AXO as expressed by comparison of the I_{50}-value of 6-phenyl-4(3*H*)-pteridinone (**6**, R = H). There is almost no difference in the inhibitory property of a linear, branched or an heteroatom containing alkyl chain.

Accurate I_{50}-data for the series of 3-alkyl-6-phenyl-4(3*H*)-pteridinone 8-oxides **5** could not be obtained [25]. They may vary between 120 μM and 200 μM. These values are certainly larger than those obtained with 6-phenyl-4(3*H*)-pteridinone 8-oxide which has a I_{50}-value of 58 ± 4 μM.

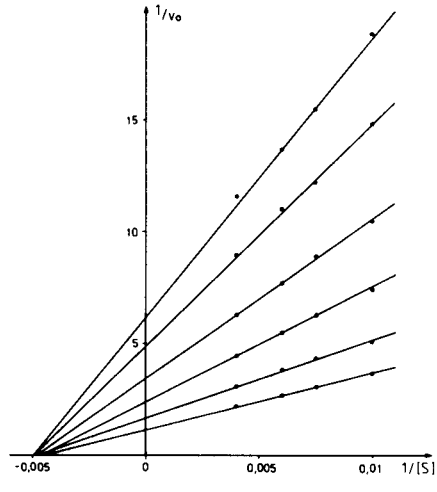


Figure 2. Reciprocal plots for the oxidation of 100 μM 1-methylxanthine and five fixed concentrations of 3-methyl-6-phenyl-4(3*H*)-pteridinone (**6a**) by AXO at *p*H = 7.25. The inhibitor concentrations used were (from bottom to top): 0, 24.9, 50.3, 59.9, 75.4 and 88.0 μM.

We selected the compounds **6a** and **6b** to study the mode of inhibition of AXO in some detail. In Figure 2 the Lineweaver-Burk plots for five concentrations (ranging from 25 to 88 μM) of 3-methyl-6-phenyl-4(3*H*)-pteridinone are drawn, using 1-methylxanthine as substrate. It was found that the inhibition probably is of the non-competitive type since the lines give a point of intersection at reci-

procal substrate concentration. This indicates that the Km value is not influenced by the inhibitor [26]. By replotting the data from Figure 2 (see Figure 3) in order to calculate the Ki-value an increase in the reciprocal value of Vm was observed at inhibitors concentrations higher than about 60 μM. From the replot using points between 0 and 50 μM, a Ki-value of about 47 μM is calculated. Clearly another phenomenon occurs at inhibitor concentrations higher than 60 μM, possibly due to a shift from the non-competitive type of inhibition to another sort of inhibition pattern, or due to chemical conversion.

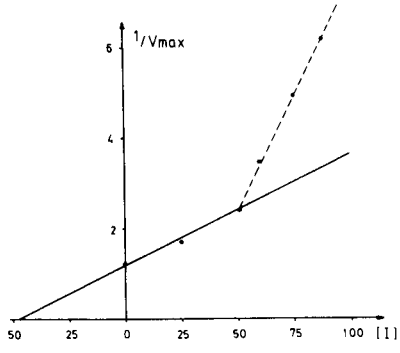


Figure 3. A replot of the applied inhbitor concentrations of 3-methyl-6-phenyl-4(3*H*)-pteridinone (**6a**) *versus* the *y* intercept (Vm^{-1}) of Figure 2.

In the case of 3-ethyl-6-phenyl-4(3*H*)-pteridinone the inhibition took a quite different course. The results of experiments with 1-methylxanthine using three concentrations of inhibitor (ranging from 25 to 77 μM) are plotted in Figure 4; the plots are parallel since both the Km and Vm value decrease with increasing inhibition concentration. This behaviour is consistent with that of uncompetitive inhibition [26,27]. From the replot (see insert Figure 4) an inhibition constant (Ki) of 19.6 μM is calculated. In contrary to the 3-methylderivative this compound is possibly not bounded in the vicinity of the active site of the enzyme but probably at another site. Apparently, the 3-ethyl derivative (partly) binds to the enzyme-substrate complex making a fraction of the offered AXO not available for oxidation of 1-methylxanthine.

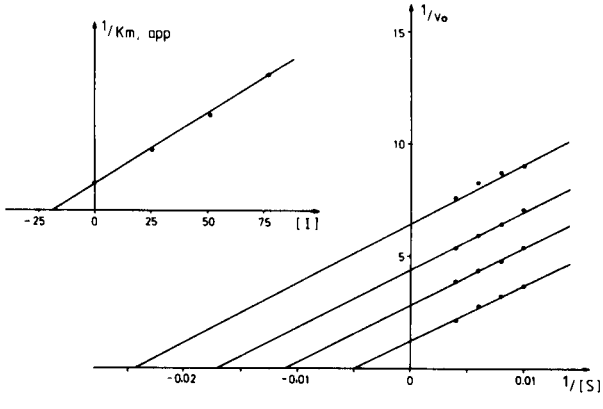


Figure 4. Reciprocal plots for the oxidation of 100 μM 1-methylxanthine and three fixed concentrations of 3-ethyl-6-phenyl-4(3*H*)-pteridinone (**6b**) by AXO at *p*H = 7.25. The inhibitor concentrations used were (from bottom to top): 0, 25.7, 51.5 and 77.2 μM. The insert shows a replot of the inhibitor concentrations **6b** *versus* the corresponding apparent Km-value.

So the conclusion can be drawn that **6a** is a non-competitive inhibitor which is involved in binding at the active site, while the 3-ethyl analogue **6b** binds at another site since in this case no oxidation product is observed to be formed.

It is possible that introduction of AXO to the assay mixture of both substrate and inhibitor has created "protective" conditions in which the active site is protected by the high concentration of substrate. This may prevent the inhibitor of binding at the active site. However after incubation of AXO at 25° with different concentrations of **6e** during thirty minutes, the actual activity was measured at 25° using 100 μM of 1-methylxanthine. No difference in I_{50}-value was observed. This observation provides additional evidence that 3-alkyl-6-phenyl-4(3*H*)-pteridinone with an alkyl group larger than methyl cannot be accommodated at the active site of the AXO.

As discussed before the compounds **5** and **6** differ in their electron density distribution. Although it is possible that apart from steric reasons the different oxidation rates of the compounds **5** and **6** with xanthine oxidase from *Arthrobacter* are due to those differences in electron density distribution, it remains however questionable whether the small but distinct differences in electron density have an important impact on these rates.

EXPERIMENTAL

Melting points are uncorrected. The ^1H nmr spectra were recorded in deuterated dimethylsulphoxide solutions on Varian EM-390 (90 MHz) spectrometer with TMS as internal standard. The mass spectra were obtained on AEJ MS-902 equipped with a VG-ZAB console. Only the data of ^1H nmr spectra not shown in Tables 1 and 2 are given. For the complex multiplet signals centers of gravity are reported and all the NH-protons were found to be exchangeable with deuterated methanol.

2-Amino-3-carbethoxy-5-phenylpyrazine 1-Oxide (**1**).

This compound was prepared as described previously [10], mp 143-145° (lit [10] 135-137°); ^1H nmr: δ 1.38 (3H, t, CH_3), 4.43 (2H, q, CH_2), 7.47 (3H, m, ArH), 7.75 (2H, br s, NH_2), 8.03 (2H, m, ArH), 9.16 (1H, s, 6-H).

2-Amino-3-carbethoxy-5-phenylpyrazine (**2**).

A stirred solution of **1** (3.00 g, 11.6 mmoles) in dry tetrahydrofuran (150 ml) maintained at 0° was treated slowly, over a period of five minutes with phosphorus trichloride (3 ml). Stirring was continued at room temperature for 30 minutes and the reaction mixture was concentrated to small volume under reduced pressure. Ice-water (300 ml) was added, the precipitate formed filtered off, washed with cold water and recrystallized from ethanol to give 2.23 g (79%) of yellowish needles, mp 89-90°. ^1H nmr: δ 1.35 (3H, t, CH_3), 4.38 (2H, q, CH_2), 7.45 (5H, br m, ArH + NH_2), 7.98 (2H, m, ArH), 8.88 (1H, s, 6-H).

Anal. Calcd. for $C_{13}H_{13}N_3O_2$ (243.26): C, 64.18; H, 5.39; N, 17.28. Found: C, 64.09; H, 5.37; N, 17.36.

General Procedure for the Aminolysis of 2-Amino-3-ethoxycarbonyl-5-phenylpyrazine 1-Oxide and 2-Amino-3-ethoxycarbonyl-5-phenylpyrazine.

A solution of **1** or **2** in the required alkylamine (**a-d**) or its 40% aqueous solution (**e-j**) was stirred for 2 hours under moderate heating (ca 80°). Then the mixture was evaporated under reduced pressure to dryness (**a-g**) or cooled (**h-j**) and the solid material recrystallized from ethanol/water (2:1) (if not stated otherwise).

2-Amino-3-(methylcarbamoyl)-5-phenylpyrazine 1-Oxide (**3a**).

This compound was prepared in a yield of 82% as yellow needles, mp 190-191°; ms: m/e 244 (M^+).

Anal. Calcd. for $C_{12}H_{12}N_4O_2$ (244.25): C, 59.00; H, 4.95. Found: C, 59.11; H, 4.85.

2-Amino-3-(ethylcarbamoyl)-5-phenylpyrazine 1-Oxide (**3b**).

This compound was prepared in a yield of 91% as yellow needles, mp 170.0-171.5°; ^1H nmr: δ 1.17 (3H, t, CH_3).

Anal. Calcd. for $C_{13}H_{14}N_4O_2$ (258.27): C, 60.45; H, 5.46. Found: C, 60.23; H, 5.16.

2-Amino-3-(*n*-propylcarbamoyl)-5-phenylpyrazine 1-Oxide (**3c**).

This compound was prepared in a yield of 65% as yellow needles, mp 150-151°; ^1H nmr: δ 0.90 (3H, t, CH_3), 1.60 (2H, m, CH_2).

Anal. Calcd. for $C_{14}H_{16}N_4O_2$ (272.30): C, 61.75; H, 5.92. Found: C, 61.51; H, 5.68.

2-Amino-3-(*n*-butylcarbamoyl)-5-phenylpyrazine 1-Oxide (**3d**).

This compound was prepared in a yield of 96% as yellow needles, mp 122-123°; ^1H nmr: δ 0.90 (3H, t, CH_3), 1.40 (4H, m, CH_2CH_2).

Anal. Calcd. for $C_{15}H_{18}N_4O_2$ (286.33): C, 62.92; H, 6.34. Found: C, 63.19; H, 6.49.

2-Amino-3-(*i*-propylcarbamoyl)-5-phenylpyrazine 1-Oxide (**3e**).

This compound was prepared in a yield of 86% as yellow needles, mp 169-170°; ^1H nmr: δ 1.23 (6H, d, $C(CH_3)_2$).

Anal. Calcd. for $C_{14}H_{16}N_4O_2$ (272.30): C, 61.75; H, 5.92. Found: C, 61.78; H, 5.84.

2-Amino-3-(*s*-butylcarbamoyl)-5-phenylpyrazine 1-Oxide (**3f**).

This compound was prepared in a yield of 80% as yellow needles, mp 118-120°; ms: m/e 286 (M^+); ^1H nmr: δ 0.87 (3H, t, CH_3), 1.22 (3H, d, 2'-CH_3), 1.58 (2H, m, CH_2).

Anal. Calcd. for $C_{15}H_{18}N_4O_2$ (286.33): C, 62.92; H, 6.34. Found: C, 62.81; H, 6.16.

2-Amino-3-(*t*-butylcarbamoyl)-5-phenylpyrazine 1-Oxide (**3g**).

This compound was prepared in a yield of 77% as a cream powder (from chloroform/methanol), mp 214-215° dec; ^1H nmr: δ 1.28 (9H, s, $C(CH_3)_3$).

Anal. Calcd. for $C_{15}H_{18}N_4O_2{*}2H_2O$ (322.36): C, 55.89; H, 6.88; N, 17.38. Found: C, 55.54; H, 6.77; N, 17.32.

2-Amino-3-(2-hydroxyethylcarbamoyl)-5-phenylpyrazine 1-Oxide (**3h**).

This compound was prepared in a yield of 72% as yellow needles, mp 173-174°; ^1H nmr: δ 4.80 (exchangeable with perdeuteriomethanol) (1H, t, OH).

Anal. Calcd. for $C_{13}H_{14}N_4O_3$ (274.27): C, 56.92; H, 5.11. Found: C, 56.64; H, 4.88.

2-Amino-3-[(1-hydroxy-2-methylpropyl-2)carbamoyl]-5-phenylpyrazine 1-Oxide (**3i**).

This compound was prepared in a yield of 59% as fine yellow needles, mp 207-208°; ^1H nmr: δ 1.40 (6H, s, $C(CH_3)_2$), 3.48 (2H, d, CH_2O).

Anal. Calcd. for $C_{15}H_{18}N_4O_3$ (302.33): C, 59.59; H, 6.00; N, 18.53. Found: C, 59.33; H, 5.91; N, 18.36.

2-Amino-3-[(1-hydroxybutyl-2)carbamoyl]-5-phenylpyrazine 1-Oxide (**3j**).

This compound was prepared in a yield of 59% as a cream powder, mp 154-155°; ms: m/e 302 (M^+); ^1H nmr: δ 0.90 (3H, t, CH_3), 1.63 (2H, m, CH_2), 3.55 (2H, m, CH_2O).

Anal. Calcd. for $C_{15}H_{18}N_4O_3$ (302.33): C, 59.59; H, 6.00; N, 18.53. Found: C, 59.64; H, 5.91; N, 18.37.

2-Amino-3-(methylcarbamoyl)-5-phenylpyrazine (**4a**).

This compound was prepared in a yield of 85% as yellow needles, mp 130-131°.

Anal. Calcd. for $C_{12}H_{12}N_4O$ (228.25): C, 63.14; H, 5.30. Found: C, 63.42; H, 5.19.

2-Amino-3-(ethylcarbamoyl)-5-phenylpyrazine (**4b**).

This compound was prepared in a yield of 84% as yellow needles, mp 122.5-123.5°; ^1H nmr: δ 1.20 (3H, t, CH_3).

Anal. Calcd. for $C_{13}H_{14}N_4O$ (242.27): C, 64.44; H, 5.82; N, 23.13. Found: C, 64.46; H, 5.82; N, 22.99.

2-Amino-3-(*n*-propylcarbamoyl)-5-phenylpyrazine (**4c**).

This compound was prepared in a yield of 91% as cream needles, mp 130-131°; ^1H nmr: δ 0.90 (3H, t, CH_3), 1.60 (2H, m, CH_2).

Anal. Calcd. for $C_{14}H_{16}N_4O$ (256.30): C, 65.60; H, 6.29; N, 21.86. Found: C, 65.51; H, 6.20; N, 21.58.

2-Amino-3-(*n*-butylcarbamoyl)-5-phenylpyrazine (**4d**).

This compound was prepared in a yield of 92% as fine yellow needles, mp 84-85°; ^1H nmr: δ 0.92 (3H, t, CH_3), 1.47 (4H, m, CH_2CH_2).

Anal. Calcd. for $C_{15}H_{18}N_4O$ (270.33): C, 66.64; H, 6.71; N, 20.73. Found: C, 66.30; H, 6.77; N, 20.85.

2-Amino-3-(*i*-propylcarbamoyl)-5-phenylpyrazine (**4e**).

This compound was prepared in a yield of 97% as cream coloured needles, mp 125-126°; ^1H nmr: δ 1.23 (6H, d, $C(CH_3)_2$).

Anal. Calcd. for $C_{14}H_{16}N_4O$ (256.30): C, 65.60; H, 6.29; N, 21.86. Found: C, 65.62; H, 6.34; N, 22.06.

2-Amino-3-(*s*-butylcarbamoyl)-5-phenylpyrazine (**4f**).

This compound was prepared in a yield of 71% as cream needles, mp 88-89°; ^1H nmr: δ 0.90 (3H, t, CH_3), 1.22 (3H, d, 2'-CH_3), 1.58 (2H, m, CH_2).

Anal. Calcd. for $C_{15}H_{18}N_4O$ (270.33): C, 66.64; H, 6.71; N, 20.73. Found: C, 66.63; H, 6.84; N, 20.88.

2-Amino-3-(*t*-butylcarbamoyl)-5-phenylpyrazine (**4g**).

This compound was prepared in a yield of 92% as a cream powder (from chloroform/methanol), mp 159-161° dec; ^1H nmr: δ 1.28 (9H, s, $C(CH_3)_3$).

Anal. Calcd. for $C_{15}H_{18}N_4O{*}2.5\ H_2O$ (315.37): C, 57.12; H, 7.35; N, 17.77. Found: C, 56.88; H, 8.03; N, 17.44.

2-Amino-3-(2-hydroxyethylcarbamoyl)-5-phenylpyrazine (**4h**).

This compound was prepared in a yield of 76% as yellow needles, mp 158-159°; ^1H nmr: δ 3.50 (4H, m, CH_2CH_2).

Anal. Calcd. for $C_{13}H_{14}N_4O_2$ (258.27): C, 60.45; H, 5.46; N, 21.69. Found: C, 60.15; H, 5.55; N, 21.73.

General Procedure for the Ring Closure of 2-Amino-3-(alkylcarbamoyl)-5-phenylpyrazine 1-Oxides (**3**) into 6-Phenyl-3-alkyl-4(3*H*)-pteridinone 8-Oxides (**5**) and of 2-Amino-3-(alkylcarbamoyl)-5-phenylpyrazine (**4**) into 6-Phenyl-3-alkyl-4(3*H*)-pteridinones (**6**).

A solution of 1.0 mmole of the pyrazine derivative in triethyl orthoformate solution (3 ml) was heated with stirring in an open flask at 145° for several hours (given below). If necessary, an additional volume of triethyl orthoformate was added. The reaction was monitored by tlc (Merck plastic sheets Silica gel 60 F_{254}, chloroform/methanol 19:1 as developing system). After the ring closure was completed the reaction mixture was cooled, the precipitate filtered off, washed with ethanol and ether and recrys-

tallized from dimethylsulphoxide-water (**5**) or chloroform-light petroleum ether (bp 40-60) (**6**).

3-Methyl-6-phenyl-4(3*H*)-pteridinone 8-Oxide (**5a**).

This compound was obtained after 8 hours in a yield of 80% as a white powder, mp 304-305° dec; ^{1}H nmr: δ 8.60 (1H, s, H-2).

Anal. Calcd. for $C_{13}H_{10}N_4O_2$ (254.24): C, 61.40; H, 3.96. Found: C, 61.48; H, 3.62.

3-Ethyl-6-phenyl-4(3*H*)-pteridinone 8-Oxide (**5b**).

This compound was obtained after 8 hours in a yield of 56% as a white powder, mp 288-290° dec; ^{1}H nmr: δ 1.32 (3H, t, CH_3), 8.63 (1H, s, H-2).

Anal. Calcd. for $C_{14}H_{12}N_4O_2$ (268.27): C, 62.68; H, 4.51. Found: C, 62.45; H, 4.29.

3-*n*-Propyl-6-phenyl-4(3*H*)-pteridinone 8-Oxide (**5c**).

This compound was obtained after 6 hours in a yield of 34% as a white powder, mp 247-249° dec; ^{1}H nmr: δ (3H, t, CH_3), 1.76 (2H, m, C_{1-2}), 8.62 (1H, s, H-2).

Anal. Calcd. for $C_{15}H_{14}N_4O_2$ (282.29): C, 63.82; H, 5.00. Found: C, 63.50; H, 4.71.

3-*n*-Butyl-6-phenyl-4(3*H*)-pteridinone 8-Oxide (**5d**).

This compound was obtained after 6 hours in a yield of 94% as a white powder, mp 230-232° dec; ^{1}H nmr: δ 0.93 (3H, t, CH_3), 1.33 (2H, m, CH_2), 1.70 (2H, m, CH_2), 8.62 (1H, s, H-2).

Anal. Calcd. for $C_{16}H_{16}N_4O_2$ (296.32): C, 64.85; H, 5.44. Found: C, 64.58; H, 5.47.

3-*i*-Propyl-6-phenyl-4(3*H*)-pteridinone 8-Oxide (**5e**).

This compound was obtained after 6 hours in a yield of 29% as a cream powder, mp 286-288° dec; ^{1}H nmr: δ 1.47 (6H, d, $C(CH_3)_2$), 8.67 (1H, s, H-2).

Anal. Calcd. for $C_{15}H_{14}N_4O_2$ (282.29): C, 63.82; H, 5.00. Found: C, 63.56; H, 4.87.

3-*s*-Butyl-6-phenyl-4(3*H*)-pteridinone 8-Oxide (**5f**).

This compound was obtained after 6 hours in a yield of 29% as a white powder, mp 231-232° dec: ^{1}H nmr: δ 0.85 (3H, t, CH_3), 1.47 (3H, d, 2'-CH_3), 1.83 (2H, m, CH_2), 8.63 (1H, s, H-2).

Anal. Calcd. for $C_{16}H_{16}N_4O_2$ (296.32): C, 64.85; H, 5.44; N, 18.91. Found: C, 64.49; H, 5.31; N, 18.86.

3-[1-(Diethoxymethyloxy)butyl-2]-6-phenyl-4(3*H*)-pteridinone 8-Oxide (**5k**).

This compound was obtained after 16 hours in a yield of 28% as a white powder, mp 131-132°; ms: m/e 414.1907 (M^+) (Calcd. 414.1903), 398.1960 (M^+-16) (Calcd. 398.1954); ^{1}H nmr: δ 0.85 (3H, t, CH_3), 1.03 (3H, t, CH_3), 1.18 (3H, t, CH_3), 1.92 (2H, m, CH_2), 3.45 (2H, q, OCH_2), 3.78 (2H, d, CH_2O), 4.15 (2H, q, OCH_2), 8.56 (1H, s, H-2), 8.80 (1H, s, $OCH(O-)_2$).

Anal. Calcd. for $C_{21}H_{26}N_4O_5$ (414.45): C, 60.86; H, 6.32; N, 13.52. Found: C, 60.50; H, 6.29; N, 13.70.

3-Methyl-6-phenyl-4(3*H*)-pteridinone (**6a**).

This compound was obtained after 6 hours in a yield of 78% as a cream powder, mp 235-236° dec; ^{1}H nmr: δ 8.65 (1H, s, H-2).

Anal. Calcd. for $C_{13}H_{10}N_4O$ (238.24): C, 65.53; H, 4.23; N, 23.52. Found: C, 65.37; H, 4.22; N, 24.07.

3-Ethyl-6-phenyl-4(3*H*)-pteridinone (**6b**).

This compound was obtained after 6 hours in a yield of 49% as a white powder, mp 215-216° dec; ms: m/e 252 (M^+); ^{1}H nmr: δ 1.33 (3H, t, CH_3), 8.70 (1H, s, H-2).

Anal. Calcd. for $C_{14}H_{12}N_4O$ (252.27): C, 66.65; H, 4.79; N, 22.21. Found: C, 66.01; H, 4.72; N, 22.24.

3-*n*-Propyl-6-phenyl-4(3*H*)-pteridinone (**6c**).

This compound was obtained after 7 hours in a yield of 71% as white prisms, mp 133-134°; ^{1}H nmr: δ 0.92 (3H, t, CH_3), 1.78 (2H, m, CH_2), 8.68 (1H, s, H-2).

Anal. Calcd. for $C_{15}H_{14}N_4O$ (266.29): C, 67.65; H, 5.30; N, 21.04. Found: C, 67.22; H, 5.36: N, 21.33.

3-*n*-Butyl-6-phenyl-4(3*H*)-pteridinone (**6d**).

This compound was obtained after 8 hours in a yield of 32% as white prisms, mp 103-104°; ^{1}H nmr: δ 0.94 (3H, t, CH_3), 1.35 (2H, m, CH_2), 1.72 (2H, m, CH_2), 8.68 (1H, s, H-2).

Anal. Calcd. for $C_{16}H_{16}N_4O$ (280.32): C, 68.55; H, 5.75; N, 19.99. Found: C, 68.24; H, 5.77; N, 20.18.

3-*i*-Propyl-6-phenyl-4(3*H*)-pteridinone (**6e**).

This compound was obtained after 7 hours in a yield of 58% as white prisms, mp 160-161°; ms: m/e 266 (M^+); ^{1}H nmr: δ 1.50 (6H, d, $C(CH_3)_2$), 8.74 (1H, s, H-2).

Anal. Calcd. for $C_{15}H_{14}N_4O$ (266.29): C, 67.65; H, 5.30; N, 21.04. Found: C, 67.44; H, 5.41; N, 21.18.

2-(Formylamino)-3-carbethoxy-5-phenylpyrazine (**7a**).

A solution of **2** (300 mg, 1.2 mmole) in triethyl orthoformate (3 ml) and acetic anhydride (6 ml) was heated under reflux at 110° for 1.5 hours. The reaction mixture was evaporated to dryness, triturated twice with ethanol and evaporated again to give 250 mg (73%) of solid material with mp 121-125°. ^{1}H nmr: δ 1.37 (3H, t, CH_3), 4.52 (2H, q, CH_2), 7.52 (3H, m, ArH), 8.08 (2H, m, ArH), 9.18 (1H, s, 6-H), 9.30 (1H, d, NCHO) (exchanged with perdeuteriomethanol into 9.28).

2-(Formylamino)-3-(*s*-butylcarbamoyl)-5-phenylpyrazine (**7b**).

The reaction mixture obtained from **4f** (270 mg, 1.0 mmole) after ten hours of heating following the general ring closure procedure, was evaporated to dryness to give 14 mg (3%) of a solid material; ^{1}H nmr: δ 0.92 (3H, t, -CH_3), 1.27 (3H, d, 2'-CH_3), 1.62 (2H, m, -CH_2-), 4.06 (1H, m, -CH-), 7.53 (3H, m, ArH), 8.25 (2H, m, ArH), 8.87 (1H, br d, NH), 9.12 (1H, s, 6-H), 9.45 (1H, s, NCHO) (exchangeable with perdeuteriomethanol) into 9.48 (1H, s).

Enzymatic Assays.

The growth of the *Arthrobacter* M-4 strain, the preparation of cell-free extract and the assay for protein and activity were performed as already described previously [4]. As storage buffer 10 m*M* of potassium phosphate (*p*H = 7.25, 0.1 m*M* EDTA) was used. Each assay was carried out at least in duplicate. Stock solutions of all the compounds were prepared in 96% of ethanol wherby each 1 m*M* was first dissolved in one millileter of 96% of ethanol and then diluted with distilled water. Those solutions where used in such a concentration range taking care that the amount of ethanol in the final assay mixture did not exceed the 5%. No inhibition of the bacterial enzyme was observed under these circumstances. Only when the amount of ethanol was higher than 5 *M* or about 20%, alterations in activity were observed using 100 μ*M* 1-methylxanthine as substrate. For the bacterial enzyme, one unit of enzyme activity is the amount of enzyme which oxidizes 1 μmol of 1-methylxanthine per minute at 25°. The assay conditions were: 100 μ*M* of substrate in 50 m*M* potassium phosphate buffer (*p*H = 7.25, containing 0.1 m*M* EDTA) using oxygen as final electron acceptor. The reaction was monitored at 292 nm (log Δε = 4.09) using a Varian DMS 100 spectrophotometer coupled with a DS 15 data station. The rate was determined from the initial slope of the absorbance *versus* time, representing the rate of appearance of the product. For determination of the I_{50}-values 100 μ*M* of 1-methylxanthine was mixed with appropriate amounts of inhibitor, ranging from 25 to 250 μ*M*. The I_{50}-value was calculated by plotting the logarithm of the inhibitor concentration *versus* the activity. The best fit was estimated by the method of linear least squares with a correlation coefficient between 0.94 and 0.99. The concentration at which 50% of the original activity was lost, was calculated using the best fit.

The detailed inhibition studies were performed using 100, 133, 167 and 250 μ*M* of 1-methylxanthine and 24.9, 50.3, 59.9, 75.4 and 88.0 μ*M* for compound **6a** (Figure 2) and using 100, 125, 167 and 250 μ*M* of stan-

dard substrate and 25.7, 51.5 and 77.2 μM in the case of compound **6b** (Figure 3).

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[24] In a few cases precipitation of the substrate occurred on performing the enzymatic oxidation. To avoid this problem stock solutions of the substrates were prepared in 96% of ethanol and diluted with distilled water in such a way that the amount of cosolvents was below 5%.

[25] Due to the low solubility of 3-alkyl-6-phenyl-4(3*H*-pteridinone 8-oxides **5**, even in a mixture of ethanol and buffer, and the fact that these compounds have their absorption maximum (294-296 nm, log $\Delta\epsilon$ = 4.38-4.41) at about the same wavelength where the conversion of 1-methylxanthine to 1-methyluric acid was measured, no accurate data could be obtained.

[26] I. H. Segel, "Enzyme Kinetics: Behaviour and Analysis of Rapid Equilibrium Steady-State Enzyme Systems", Wiley-Interscience, New York, 1975, p 125.

[27] 8-Bromoxanthine is an uncompetitive inhibitor of bovine milk xanthine oxidase with respect to xanthine [28]. Despite the presence of the bulky bromo group this compound interacts with the molybdenum center of xanthine oxidase.

[28] R. Hille and R. C. Stewart, *J. Biol. Chem.*, **259**, 1570 (1984).