SYNTHESIS AND OXIDATION BY XANTHINE OXIDASE FROM ARTHROBACTER M-4

OF

6-ARYL-4(3H)-PTERIDINONES AND RELATED COMPOUNDS

Why trouble to make compounds yourself
when a bug will do it for you?
(J.B.S. Haldane, 1929)

Voor Tineke

Promotor: Dr. H.C. van der Plas, hoogleraar in de organische scheikunde

J.W.G. De Meester

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
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Je voudrais aussi remercier dr. Antonio Nazaré-Pereira pour ses efforts d'augmenter l'activité de l'*Arthrobacter M-4*.

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STELLINGEN

1 Het feit dat verscheidene auteurs de recovery aan mutageniteit bij de fractionering van een monster extract bepalen door de som van de mutageniteit per fractie te vergelijken met die van het oorspronkelijke extract, geeft blijk van een weinig doordachte wijze van experimenteren.

W.R. Harris, E.K. Chess, D. Okamato, J.F. Remsen en D.W. Later, *Environment Mutagenesis*, **6**, 131 (1984).
J. Siak, T.L. Chan, T.L. Gibson en G.T. Wolff, *Atmospheric Environment*, **19**, 369 (1985).
Y. Manabe, T. Kinouchi en Y. Ohnishi, *Mutation Research*, **158**, 3 (1985).

2 De keuze van *p*-nitrobenzylbenzoaat om een onderscheid te kunnen maken tussen het acylkation en het carboxy-radicaal als intermediair in de oxidatie van benzylesters door ceriumammoniumnitraat is niet gefundeerd.

A.K. Bag, S.R. Gupta en D.N. Dhar, *Ind. Journ. Chem.*, **25B**, 433 (1986)

3 Bose *et al.* houden ten onrechte geen rekening met het feit dat gehinderde rotatie rond de Pt-N bindingen in cis-bis(nucleotide)platina verbindingen leidt tot de vorming van twee of meer rotameren, die verschillende nmr-spectra geven.

R.B. Bose, R.D. Cornelius en R.E. Viola, *J. Am. Chem. Soc.*, **108**, 4403 (1986).

4 De gronden waarop in een aantal onderzoeken aan modelverbindingen voor fotosynthese ladingsoverdracht wordt aangenomen, zijn onvoldoende.

J.R. Bolton, T.-F. Ho, S. Liauw, A. Siemiarczuk, C.S.K. Wan en A.C. Sheldon, *J. Chem. Soc., Chem. Comm.*, **1985**, 559.
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5 Fadda *et al.* suggereren ten onrechte dat in de reactie van N-ethyl-3-cyanopyridiniumjodide in waterig ethylamine geen transaminering optreedt.

A.A. Fadda, F.M. Abdelrazek en M.M. El-Habbal, *Ind. Journ. Chem.*, **25B**, 194 (1986).

6 De verklaring die Young en Chang geven voor het feit dat in deuteriochloroform het nmr-spectrum van trans-5,15-bis-[o-(*p*-tert-butyl-benzamido)phenyl]-2,8,12,18-tetra-ethyl-3,7,13,17-tetra-methylporfyrine een singlet vertoont voor de fenylprotonen van de benzamido groep en in de cis-verbinding een kwartet, is uitermate onwaarschijnlijk.

R. Young en C.K. Chang, *J. Am. Chem. Soc.*, **107**, 898 (1985).

7 Het mechanisme voor de ringsluiting van 6-arylamino-1,3-dimethyluracil met koolstofdisulfide, voorgesteld door Tominaga *et al.* is aan bedenkingen onderhevig.

Y. Tominaga, H. Okuda, M. Tochiki, Y. Matsuda en G. Kobayashi, *Heterocycles*, **15**, 679 (1981).

8 Het verschil in substraatspecificiteit tussen aldehyde oxidase en xanthine oxidase kan worden gebruikt om op enzymatische wijze verschillende produkten te bereiden zoals voorbeeldig wordt gedemonstreerd door het metabolisme van 6-deoxyacyclovir in konijnen. Bovenstaand beschreven onderzoek is tevens een goed voorbeeld van downstream processing.

T.A. Krenitsky, W.W. Hall, P. de Miranda, L.M. Beauchamp, H.J. Schaeffer en P.D. Whiteman, *Proc. Natl. Acad. Sci. USA*, **81**, 3209 (1984).

9 Het is merkwaardig dat, terwijl de Nederlandse regeringen met instemming van beide kamers voor steeds meer beroepen een pensionering op 65-jarige leeftijd wettelijk verplicht hebben gesteld, zij deze verplichte pensionering nog niet nodig achten voor de verantwoordelijke functie van minister of kamerlid.

J.W.G. De Meester

Wageningen, 24 april 1987

Synthesis and oxidation by xanthine oxidase from Arthrobacter M-4 of 6-aryl-4(3H)-pteridinones and related compounds.

In het kader van hun stage of doctoraalvak hebben Ineke van der Hoef, Peter Assink, Paul Corstjens, Ron Ogg en Andries Warreman diverse nieuwe ideeën verder uitgezocht.

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CONTENTS

Parts of this thesis have been published separately

Chapter 6 H. Sladowska, J.W.G. De Meester and H.C. van der Plas, *J. Heterocyclic Chem.*, **21**, 477-481 (1986)

Chapter 7 J.W.G. De Meester, W. Kraus, W.J. Middelhoven and H.C. van der Plas, *Bio-Organic Heterocycles 1986 - Synthesis, Mechanisms and Bioactivity*, Proceedings of the Fourth FECHEM Conference on Heterocycles in Bio-Organic Chemistry, Elsevier, Amsterdam, p. 243-251 (1986).

Chapter 2 and 5 are in press

Chapter 2 J.W.G. De Meester, W.J. Middelhoven and H.C. van der Plas, *J. Heterocyclic Chem.* (1987).

Chapter 5 H.J. Brons, M.W. Breedveld, W.J. Middelhoven, F. Muller, J.W.G. De Meester and H.C. van der Plas, *Biotechnol. Appl. Biochem.* (1987).

Chapters 3 and 4 are submitted for publication

Chapter 3 J.W.G. De Meester, H.C. van der Plas, H.J. Brons and W.J. Middelhoven, *J. Heterocyclic Chem.*

Chapter 4 J.W.G. De Meester, W. Kraus, H.C. van der Plas, H.J. Brons and W.J. Middelboven, *J. Heterocyclic Chem.*

1 INTRODUCTION

1.1 GENERAL

The introduction of functional groups in azaheterocycles by the use of (immobilized) enzymes and bacterial cells has been a research subject [1] in the Department of Organic Chemistry since 1975. Bovine milk xanthine oxidase [2-4] was selected as the first enzyme to be studied because of its easy availability and its well documented broad substrate specificity towards azaheteroaromatics, although bovine milk xanthine oxidase showed substrate inhibition towards xanthine [2,5,6]. A number of 7-aryl-4(3*H*)-pteridinones were found to be converted with ease into their corresponding lumazine derivatives [7].

Later, xanthine dehydrogenase from chicken liver [8] was also succesfully immobilized and demonstrated to be useful in small scale preparative oxidations. As an immobilized enzyme could be considered as a model for an enzyme in its natural environment a quantitative structure-activity relationship (QSAR) study of the inhibitory properties of 6-aryl-4(3*H*)-pteridinones on the oxidation of xanthine into uric acid by immobilized bovine milk xanthine oxidase [9] contributed to a better understanding of the shape of the active site of the immobilized xanthine oxidase [10].

Recently a comprehensive study appeared on various immobilization procedures for rabbit liver aldehyde oxidase [11,12] which catalyzes the oxidation of N-alkyl-, N-arylazinium salts [13-15] and pyrimidine derivatives [16]. This enzyme was found to possess a markedly different substrate specificity compared to xanthine oxidase and xanthine dehydrogenase [17].

In 1978 xanthine oxidase was isolated from *Arthrobacter* S-2 [18]. It was reported to show substrate activation rather than substrate inhibition and to have a much higher specific activity than the milk enzyme. Because of these interesting properties xanthine oxidase from *Arthrobacter* X-4 has been studied for potential application as biocatalyst [19]. Due to the high phosphate requirement for growing of *Arthrobacter* X-4 and S-2 strains our attention was soon turned towards bacterial xanthine oxidase from another strain *i.e.* *Arthrobacter* M-4. This thesis present a study on the substrate specificity and the potential use in immobilized form of xanthine oxidase from *Arthrobacter* M-4.

1.2 IMMOBILIZED ENZYMES AND BACTERIAL CELLS IN SYNTHETIC ORGANIC CHEMISTRY

Enzymes are able to catalyze chemical reactions in the living cells. They possess a unique reaction specificity, have in general a high turnover number and can operate under mild conditions *i.e.* moderate temperatures and atmospheric pressure in an aqueous environment. An important criterium for selecting an enzyme for application in organic chemistry is its ability to catalyze reactions which are chemically difficult or impossible to execute. Although during the last decades the potential of enzymes to carry out unusual reactions on a preparative scale is recognized [20] there are serious drawbacks to apply them very generally. Enzymes are present in tissues, cells and plants usually in very low concentrations. Isolation of enzymes in sufficient large amounts is often expensive, time-consuming and lowers frequently their stability. An efficient utilization of enzymes is further obscured by the low recovery when enzymatic reactions are performed in aqueous solutions.

Immobilization of enzymes on a solid support [21] proved to be a promising technique to cope with most of above mentioned problems. Immobilized enzymes have important advantages over soluble enzymes : they can repeatedly be used, their stability is enhanced through multi-point attachment, they can be used as a second phase in an continuous reaction, product(s) are usually obtained in a high state of purity, they can be "tailor-made" for specific purposes and finally, they require less labour and a minimal work-up.

The use of whole micro-organisms instead of "pure" enzymes has supplementary advantages [22,23]. By changing the growth conditions, manipulation of the biochemical regulation systems, recombinant DNA technology or genetic engineering the amount and activity of a certain enzyme could be increased. Instead of isolation of the desired enzyme the whole microbial cells can be immobilized enhancing the final stability through partial or complete retention of the whole enzymatic system.

The introduction of immobilized enzymes and cells in the past fifteen years in organic synthesis has steadily advanced. General criteria for the characterization of an immobilized biocatalyst have been formulated [24]. The availability and documentation of purified enzymes have been improved and many immobilization procedures using various suppports and techniques were described [21,23,25-27]. Furthermore, the integration of both enzymatic and non-enzymatic reactions in the preparation of organic compounds is actual today [28,29]. At present special fields of interest for the use of immobilized enzymes and cells are found in the preparation of steroids, antibiotics [30], optically pure

amino acids and peptides [30-34]. Nowadays more attention is paid to plant cells [35,36] and to the introduction of enzymes in non-aqueous systems [37,38] and in reversed micelles [39,40].

Although the theoretical knowledge of enzyme immobilization and stabilization is steadily advancing, the choice which of the support/immobilization method combinations for a specific enzyme or micro-organism is the best, is still mainly a matter of trial and error. Furthermore, this choice will be dictated by the demands set by the system under study [21].

1.3 BACTERIAL XANTHINE OXIDASES

Xanthine oxidase (E.C. 1.1.3.22) [41], xanthine dehydrogenase (E.C. 1.1.1.204) [41] and aldehyde oxidase (E.C. 1.2.3.1) [41] are closely related iron-sulfur containing flavomolybdoproteins. The reaction they catalyze can formally be represented as a hydroxylation.

$$RH + H_2O \longrightarrow ROH + 2e^- + 2H^+$$

In the above mentioned reaction RH is the reducing substrate. The oxygen introduced into RH is derived from water and not from molecular oxygen as demonstrated with isotopicallly labelled oxygen and water [42]. Although with xanthine dehydrogenases the same reaction can be performed the difference between oxidases and dehydrogenases is related to their final electron acceptor: the former preferentially uses molecular oxygen giving rise to H_2O_2, while the latter needs NAD^+ (or other oxidizing substrates) as the final electron acceptor, thus yielding NADH. Under both aerobic and unaerobic conditions [43,44] other compounds too can act as final electron acceptor, but their reactivity or efficiency widely differs.

Many bacterial xanthine dehydrogenases [45-54] are isolated and characterized. From the resulting NADH energy under the form of ATP can be stored by these micro-organisms [49]. Furthermore, the xanthine dehydrogenase activity is usually higher compared to xanthine oxidase activity [49,55]. Xanthine dehydrogenase is not so attractive for the preparation of hydroxylated compounds, because - as we have seen - NAD^+ [48,51-53] or an artificial electron acceptor [45-47,50,54] must be added to complete the reaction. This usually requires cofactor regeneration even when immobilized systems are used [56] and a more laborious work-up. Because of the above mentioned reasons xanthine oxidase simply using molecular oxygen is easier to apply.

Compared to the publications concerning xanthine dehydrogenase [45-54] only a few reports on bacterial xanthine oxidases [18,19,50,55] are available covering the last ten years. Woolfolk and Downard [50] screened about fifty bacterial strains containing enzymes which are able to oxidize xanthine into uric acid. Aerobic grown *Arthrobacter* and *Nocardia* strains utilize molecular oxygen relatively efficient. Since the *Arthrobacter* strains gave by far the highest specific activities with molecular oxygen and did not use NAD^+ as electron acceptor, xantine oxidase from *Arthrobacter* S-2 was isolated and some of its basic features examined [18]. The molecular weight of xanthine oxidase from *Arthrobacter* S-2 is 146 000 and contains a subunit of 79 000 indicating that this enzyme has a dimeric structure. Also the xanthine oxidase isolated from *Enterobacter cloacae* is a dimer with a molecular weight of 128 000 and 69 000 for the subunit [55]. Both oxidases have an absorption spectrum which is quite similar to that of milk xanthine oxidase, indicating that the prosthetic group is almost the same. Since the A_{280}/A_{450} ratio is about half of which is given for bovine milk xanthine oxidase [18], the protein content per prosthetic group is about half that of the milk enzyme, which molecular weight varies according to the literature from 283 000 [57] to 362 000 [58].

The bacterial xanthine oxidase also consists of two independent subunits and contains probably one molybdenum atom, one molecule of flavin and two Fe/S centres per subunit. As in aldehyde oxidase and in bovine milk xanthine oxidase the molybdenum of the bacterial enzyme is probably also bound to a cofactor [59], which is non-covalently bound to protein [60]. Recently this cofactor [61] has been shown to be a pterin in tetrahydroform [62,63]. Two stable fluorescent derivatives of this organic compound have been structurally characterized, revealing that the side chain at C-6 contains two sulfur atoms and a phosphate group [64,65]. One of these compounds is strongly related to urothione, suggesting also a metabolic relationship between the molybdenum cofactor and urothione [66]. Since this pterin cofactor is isolated from different molybdoenzymes from different sources including liver tissues and bacteria, the general occurrence of this molybdopterin [60,65] is suggested.

1.4 MECHANISM OF THE OXIDATION

A kinetic model presented by Olson and coworkers [67] for the catalysis of xanthine oxidase explains the oxidation of xanthine into uric acid by an initial attack of a disulfide group at C-8 (Figure 1.1). Rehybridization of the sp^3-carbon to a sp^2-carbon takes place after proton abstraction which releases

the electron pair on N-7 resulting into Mo(IV). The disulfide linkage in the reaction intermediate is prone to attack by water, resulting in uric acid. The electron pair in Mo(IV) is transferred via flavin (FAD) to the final electron acceptor. Many proposals have been made on both the nature of the proton acceptor [43,68,69] and the nucleophilic species [68-71] but they did not essentially alter the above described model.

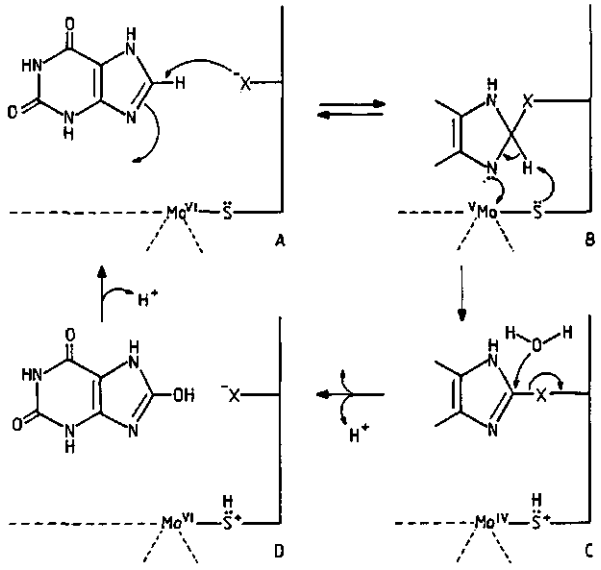


Figure 1.1 Schematic representation of xanthine by xanthine oxidase as proposed by Olson et al. [67].

In view of the recent elucidation of the structure of the pterin cofactor the kinetic model originally presented by Olson is extended by Robins and his coworkers (Figure 1.2) [72]. Very important here is the fact that the enzymatic nucleophile and the molybdenum atom in the active site are brought together in the pterin cofactor. The proposal of Robins is also taken as a fair approximation of the mechanism in the bacterial enzyme because of the close relationship in structure between xanthine oxidase from milk and that from *Arthrobacter*.

In this model the xanthine oxidase molybdenum cofactor binds xanthine at O-6 and N-7 to give a so-called *Type I Binding* [72]. The first step is facilitated by the enhanced electrophilicity at C-8. The two subsequent steps are essentially the same as in the model of Olson [67]. In a somewhat similar way the process of formation of xanthine from hypoxanthine is visualized to take place via the molybdenum cofactor binding at N-9 and N-3 to the substrate in a

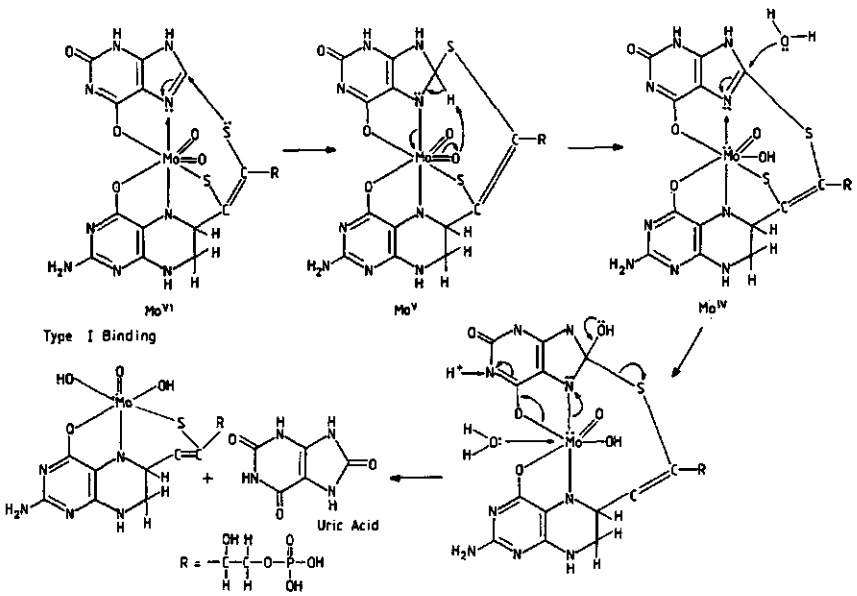


Figure 1.2 Representation of the binding pattern of xanthine with molybdopterin and the oxidation into uric acid by xanthine oxidase as proposed by Robins et al. [72].

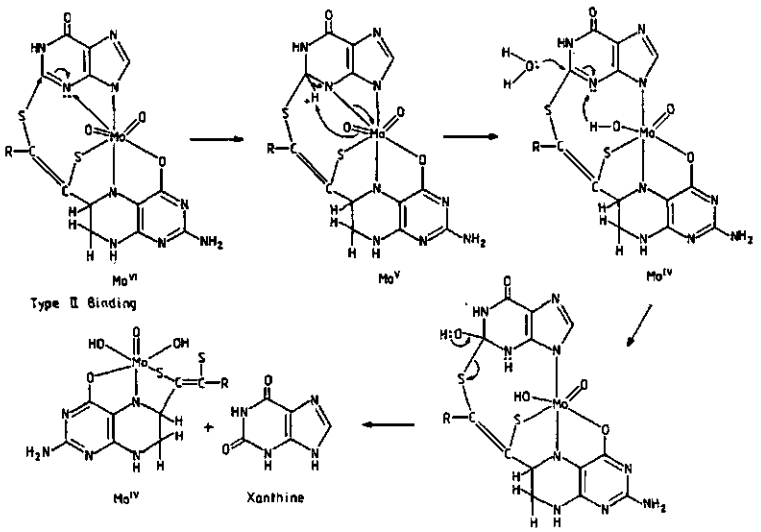
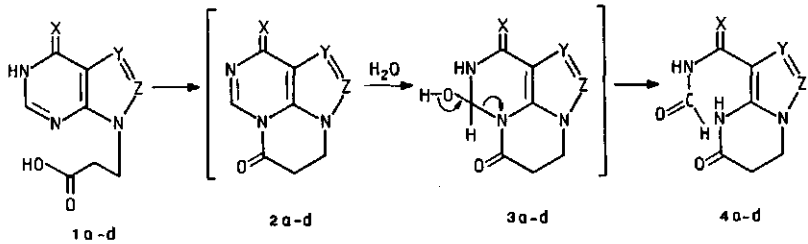


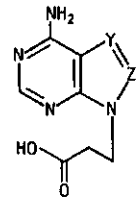
Figure 1.3 Representation of the oxidation of hypoxanthine into xanthine by xanthine oxidase as proposed by Robins et al. [72].

binding pattern which is called by Robins a *Type II Binding* (Figure 1.3). Also from this figure it is clear that molybdenum enhances the electrophilicity at C-2 of hypoxanthine.

Bergmann and Levene [73] have postulated that induction of a tautomeric shift of the H-atom at N-1 of hypoxanthine to N-3 by an acceptor group at the active site of xanthine oxidase, leading to a paraquinoid structure, is an important feature of the oxidation process. This postulate of tautomeric shifts was used to explain the experimental observed large differences in oxidation rate between purine [73] and pteridine [74,75] derivatives after N-methylation at different positions.



	X	Y	Z
a	O	CH	N
b	O	CH	CH
c	O	N	CH
d	S	N	CH



	Y	Z
a	CH	CH
b	CH	N

Scheme 1.1

The importance of a paraquinoid structure for easy nucleophilic attack at C-2 in hypoxanthine derivatives is also supported by recent experiments carried out by Seela and Rosemeyer [76-78]. They found that reaction of compounds **1a-1d** with a water soluble carbodiimide derivative in aqueous dioxane leads to the precipitation of ring opened products **4a-4d**. According to Scheme 1.1 the activated intermediates of the acids **1a-1d** undergo an intramolecular "dehydratation" forming the tricyclic N-acyl derivatives **2a-2d**. These intermediates possess a paraquinoid structure with a positively charged carbon between the two nitrogens of the pyrimidine ring [76,77]. This enforces a spontaneous addition of a water molecule across the N(1)-C(2) bond under formation of **3a-3d**. Due to the electron withdrawing effect of the carbonyl group in the lactam ring compounds **3a-3d** undergo a spontaneous pyrimidine ring opening resulting in the formation of compounds **4a-4d**.

Kinetic studies concerning this reaction have revealed that (i) when an amino group is present at C-6 (*i.e.* **5**), no "dehydration" occurs [78] after addition of the carbodiimide, (ii) the presence of a C=S group at C-6 extremely slows down the formation of products **4d**, (iii) the influence of the five-membered ring is neglectable. The high electron-withdrawing capacity of the thione group at C-6 lowers the nucleophilicity of the pyrimidine nitrogen which attacks the activated ester, but on the other hand, also enhances the addition of water at C-2. The observed slow reaction of **1d** to **4d** seems to indicate that the intramolecular acylation is the rate-limiting step [78].

The coherency of the above developed models strongly indicate the central role of molybdenum as is also supported by the formation of catalytically important charge-transfer complexes when pteridine substrates react with the active site molybdenum [79].

1.5 OUTLINE OF THE THESIS

In general the hydroxylation of purines [18,19,55] and pteridines [18] catalyzed by xanthine oxidase from bacterial sources is scarcely documented and very little information is available on the factors determining the site of oxidation. This study was initiated to enlighten the steric and electronic effects of alkyl groups and para substituted phenyl groups in different positions of the pteridine system on the site of oxidation in these compounds.

In chapter 2 the oxidation of 6-aryl-4(3*H*)-pteridinones and 7-aryl-4(3*H*)-pteridinones containing an electron-donating substituent at the para position of the phenyl group is investigated especially in comparison with the parent system 4(3*H*)-pteridinone [80].

Both series of compounds are extended in chapter 3 by studying the influence of electron-withdrawing groups at the para position of the phenyl group. Important information is presented on the rate-limiting step in the oxidation mechanism of bacterial xanthine oxidase [81].

The preparation and reactivity of 3-alkyl-6-phenyl-4(3*H*)-pteridinones and their 8-oxides towards the bacterial xanthine oxidase is presented in chapter 4 [82].

In chapter 5 the behaviour of different inhibitors towards the highly purified bacterial enzyme from *Arthrobacter* M-4 is compared with purified bovine milk xanthine oxidase [83].

Chapter 6 deals with covalent amination of substituted aryl derivatives of pteridines and their conversion into the corresponding amino pteridines [84]

using the recent developed liquid ammonia/potassium permanganate reagent [85,86].

Finally, a comparison between the action of bovine milk xanthine oxidase and bacterial xanthine oxidase from *Arthrobacter* M-4 towards different purines and pteridines is made. A general discussion on the active site of the bacterial enzyme based on the work of this thesis supplemented with miscellaneous results is presented in chapter 7 [87].

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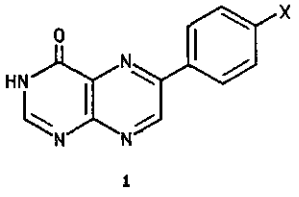
2 THE OXIDATION OF 6-ARYL-4(3H)-PTERIDINONES AND 7-ARYL-4(3H)-PTERIDINONES BY IMMOBILIZED ARTHROBACTER M-4 CELLS CONTAINING XANTHINE OXIDASE

2.1 INTRODUCTION

For many years there are ongoing studies in the laboratory of Organic Chemistry at Wageningen on the potential application of enzymes for functionalisation of azaheteroaromatics [1-9]. Enzymes under study are xanthine oxidase [3], xanthine dehydrogenase [4], aldehyde oxidase [1,2,5] and more recently chloroperoxidase [6]. Our studies so far were mainly concentrated on the use of (immobilized) xanthine oxidase isolated from bovine milk [7] (MXO) and xanthine oxidase present in *Arthrobacter* X-4 [8] and M-4 [9] bacterial cells (AXO).

Interesting differences between MXO and AXO [8,10] are observed when comparing the reaction of these enzymes with 6-phenyl-(**1a**) and 7-phenyl-4(3*H*)pteridinone (**2a**). Whereas **2a** reacts smoothly with MXO into 7-phenyllumazine [11] the 6-phenyl isomer **1a** is nearly inactive. On the contrary AXO reacts with both pteridines **1a** and **2a** [8] although **1a** reacts much faster than **2a**. The low reactivity of **1a** towards MXO cannot be ascribed to a lack of binding to the enzyme, since **1a** is found to be a very effective inhibitor of the MXO-catalyzed conversion of xanthine into uric acid. The affinity of MXO towards 7-aryl-4(3*H*)-pteridinones is about two orders of magnitude higher in comparison with that of the 7-alkyl-4(3*H*)-pteridinones [12]. This result is explained by assuming the existence of an interaction between the aryl group and hydrophobic groups present in the vicinity of the active centre. Recent QSAR studies on the inhibition of the MXO-mediated reaction of xanthine into uric acid by 6-aryl-4(3*H*)-pteridinones confirm these observations [13].

The fact that **1a** is more reactive in the AXO-mediated oxidation than **2a**, induced us to study in more detail the influence of para substituents in the C-6 phenyl ring on oxidation with *Arthrobacter* M-4 cells [14]. For that purpose we synthesized a few 6-(*p*X-phenyl)-4(3*H*)-pteridinones (X=H, (**1a**), X=CH_3, (**1b**), X=OCH_3, (**1c**)) and studied the product formation. For reasons of comparison also the 7-aryl-4(3*H*)-pteridinones (**2a-c**) were synthesized and subjected to treatment with the *Arthrobacter* M-4 cells (see Scheme 2.1). The kinetic parameters Vm and Km of the oxidation of **1a** and **2a** were determined and compared with those of 4(3*H*)-pteridinone (**15**).



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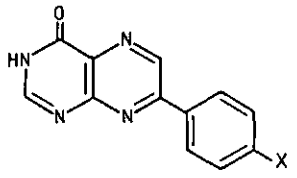


2

	a	b	c
X	H	CH_3	OCH_3

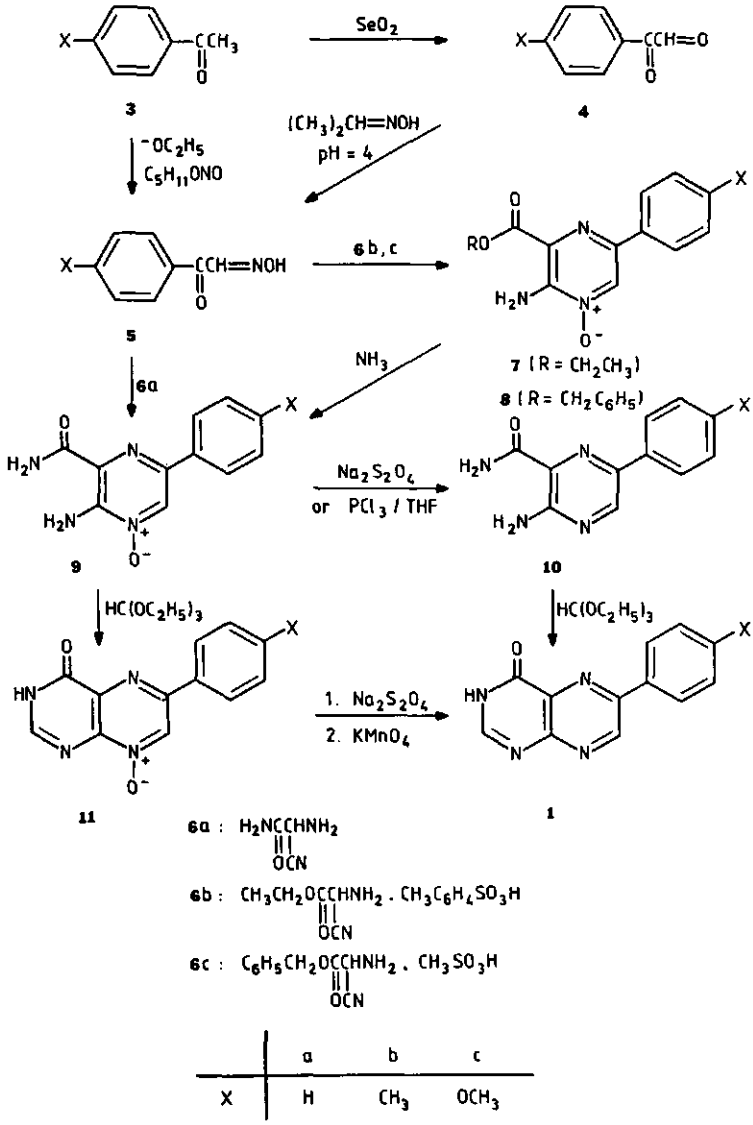
Scheme 2.1

2.2 SYNTHESIS OF 6-ARYL-4(3*H*)-PTERIDINONES AND 7-ARYL-4(3*H*)-PTERIDINONES

A general approach for synthesizing pteridines is the Gabriel-Isay procedure [15] in which a 4,5-diaminopyrimidine reacts with 1,2-dicarbonyl compounds. 4(3*H*)-Pteridinones in which either position 6 or 7 carries an aryl substituent, requires condensation of a 4,5-diamino-6(1*H*)-pyrimidinone with arylglyoxal. However, the reaction usually results in a mixture of both isomeric 6- and 7-arylpteridinones [11,15a,16,17] although by changing the acidity of the reaction medium the ratio of these two compounds can be influenced [13].

Condensation of 4,5-diamino-6(1*H*)-pyrimidinone with phenylglyoxal at pH=7.5 gives almost exclusively the 7-phenyl compound **2a**; by recrystallization from dimethylsulfoxide pure **2a** could be obtained. In a similar way the 7-aryl compounds **2b** and **2c** are prepared. Performing the condensation at pH=2.7 results in a mixture of **1a** (70%) and **2a** (30%) [13]. The separation of **1a** from **2a** is, however, difficult, time-consuming, and it lowers the yield considerably. Since in our experiments we wanted to dispose of 6-aryl-4(3*H*)-pteridinones (**1**) not contaminated with the 7-aryl-4(3*H*)-pteridinones (**2**) we turned to the versatile synthetic route [17] designed by Taylor and co-workers, which fully served our purpose. The reaction scheme is outlined in Scheme 2.2.

The synthesis required arylglyoxal-2-oximes (**5**) which were obtained by a selenium dioxide oxidation of the para substituted acetophenones (**3**) [18] and subsequent transoximation of the arylglyoxals (**4**) with acetonoxime. The best results were obtained when compounds **4** were immediately used after preparation; if necessary to keep them for some time, they were converted into their hydrates [19]. Direct preparation of **5** from **3** by a base-catalyzed reaction with



6a : $H_2NCCHNH_2$ (OCN)

6b : $CH_3CH_2OCCHNH_2 \cdot CH_3C_6H_4SO_3H$ (OCN)

6c : $C_6H_5CH_2OCCHNH_2 \cdot CH_3SO_3H$ (OCN)

	a	b	c
X	H	CH_3	OCH_3

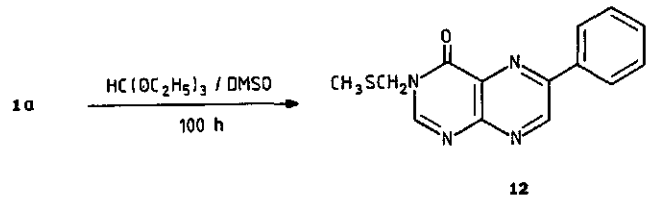
Scheme 2.2

sodium ethoxide and pentylnitrite [21] proved to be tedious and unpredictable in our hands: the yields were consistently poor, the reaction time depended on the para substituent, and variable amounts of the corresponding benzoic acid [21b] were formed. Oximation of **4** with hydroxylamine instead of acetonoxime usually resulted in the formation of the dioxime of the arylglyoxals as the main product [22]. Acetonoxime was found to be efficient and highly selective in producing the desired compounds **5** in good, reproducible yields [24].

Condensation of **5a** with 2-amino-2-cyanoaceetamide (**6a**) [25,26] (molar ratio 1:3) in glacial acetic acid for 15 hours afforded 2-amino-3-carbamoyl-5-phenylpyrazine-1-oxide (**9a**, 50%). In a very similar way compound **9c** was obtained in 23% yield. Prolonged stirring at room temperature (168 hours) resulted in somewhat higher yields, but in a less pure product which required a more rigorous work-up. Sublimation *in vacuo* of the crude pyrazine-1-oxides **9** always resulted in partial deoxygenation. A more satisfactory synthesis of the pyrazine-1-oxides **9** was accomplished by treatment of 2-amino-3-ethoxy(benzyloxy)carbonyl-5-(*p*X-phenyl)pyrazine-1-oxides (**7**, **8**) with liquid ammonia [27] or with ammonia-saturated 1-propanol producing **9**, in both cases virtually in quantitative yield. The esters **7** and **8** were formed by treatment of compounds **5** with the α-aminonitriles **6b** and **6c** in absolute methanol (70-90%).

Two methods were applied to convert **9** into the desired 6-aryl-4(3*H*)-pteridinones **1** [17] as indicated in Scheme 2.2. Reaction of **9** with triethyl orthoformate in dimethylformamide or dimethylaceetamide yielded the 6-aryl-4(3*H*)-pteridinone-8-oxides (**11**). The yields in dimethylformamide were found to be higher than in dimethylaceetamide. Treatment of **11** with sodium dithionite and subsequent mild oxidation with potassium permanganate gave the expected pteridinones **1**. Oxidation by potassium permanganate after dithionite treatment is necessary, since dithionite not only performs deoxygenation, but also reduces the pyrazine ring, as indicated by mass spectrometric data. Alternatively, **9** could first be deoxygenated by either sodium dithionite or phosphorus trichloride into the 2-amino-3-carbamoyl-5-(*p*X-phenyl)pyrazines (**10**) followed by heating with triethyl orthoformate in acetic anhydride at 150° to achieve cyclization into **1**. The reduction of **9** into **10** by treatment with boiling water containing sodium dithionite gives higher yields than the reaction with phosphorus trichloride, although the former method requires 24 hours of reaction time while only two hours are needed in the latter case. It has been found that, when the cyclization of **10a** into **1a** was not performed in acetic anhydride but in dimethylsulfoxide under strenuous conditions, besides cyclization a methylthiomethyl group is introduced at N-3: reacting **10a** with triethyl

orthoformate at 110° in dimethylsulfoxide for 16 hours gave only partial cyclization into **1a**, but by prolonging the reaction time from 16 to 100 hours 3-methylthiomethyl-6-phenyl-4(3*H*)-pteridinone (**12**) was formed in 64% yield. Structure **12** was assigned by nmr spectroscopy and microanalysis. The methylthiomethylation most probably proceeds after ring-closure into **1a**; the reaction involves the methylenesulfonium cation [28] (Scheme 2.3).



12

Scheme 2.3

In the ^{1}H nmr spectra of the pure 6- and 7-aryl-4(3*H*)-pteridinones the chemical shift of the proton at C-7 in **1** was found to be about 0.2 ppm more upfield than the proton at C-6 in **2** [13]. The difference in the ^{13}C chemical shift of C-6 and C-7 in **1** is about 3 ppm while interestingly the difference in chemical shift of C-6 and C-7 in the 7-arylderivative **2** is about 13 to 14 ppm. In Table 2.1 the chemical shifts of the 6- and 7-arylderivatives are compared with those of 4(3*H*)-pteridinone (**15**) indicating the effect of the arylgroup on the resonances of both C-6 and C-7. Similar differences were also observed before [13] confirming the structures of the compounds **1** and **2**.

*Table 2.1 ^{13}C Nmr data of 6- and 7-aryl-4(3H)-pteridinones **1a-c** and **2a-c**.*

Compound	C-2	C-4	C-6	C-7	C-9	C-10
15	149.3	160.3	144.5	150.1	155.4	134.7
1a	149.0	160.7	150.6	147.8	154.4	133.6
1b	148.6	160.4	150.4	147.4	154.0	133.4
1c	148.5	160.7	150.5	147.3	153.8	133.4
2a	149.8	160.3	142.0	154.9	155.9	133.0
2b	149.8	160.4	141.8	154.9	155.8	132.7
2c	149.6	160.3	141.5	154.9	155.6	132.1

In the pteridine-8-oxides **11** the chemical shift of H-7 has shifted about 0.25 ppm upfield relative to H-7 in **1**, but remarkably in the pyrazine-1-oxides **9** the chemical shift of H-6 has moved 0.25 ppm downfield in comparison with H-6 in the pyrazines **10**. Mass spectra of the compounds **1** and **2** also revealed disparities. In the spectra of **1a-c** a peak for $(M-27)^+$ is found, while in the spectra of **2a-c** always a peak for M^+=121 is present. The spectra of both the 6- and 7-aryl-derivatives show a peak corresponding with $(M-120)^+$.

Introduction of an electron-donating substituent at the para position of the phenylring resulted in a bathochromic shift in the UV-spectra of the pteridines. A bathochromic shift of about 25 nm resulted from the introduction of the N-oxide in the pteridine nucleus (Table 2.2).

2.3 ENZYMATIC OXIDATION

The production and characterization of *Arthrobacter* X-4 cells has already been published [8]. In a similar way *Arthrobacter* M-4 cells were grown [9]. Before using the cells, they were disrupted by ultrasonification and after centrifugation the supernatant contained the crude AXO enzyme extract. Incubation of 6-phenyl-4(3*H*)-pteridinone (**1a**) with this extract and following the reaction in time by scanning the UV-spectrum of the solution at regular intervals, resulted in a spectrum with sharp isosbestic points (Figure 2.1).

When the UV-spectrum of the reaction mixture did not change anymore, the UV-spectrum obtained was identical to that of 6-phenyllumazine (**13a**), as shown by comparison with a specimen prepared independently [30]. Further incubation of the solution containing **13a** with an additional aliquot of bacterial xanthine oxidase gave no further changes in the UV-spectrum indicating that **13a** is stable towards further oxidation. These results indicate that in the enzymatic oxidation of **1a** only one product and no by-products are formed. This result is not unexpected since the carbon atom between two ring nitrogens in pteridines and purines is highly susceptible [11,31,32] to enzymatic oxidation and the unoccupied C-7 position in **1a** is sterically hindered by the phenyl group at C-6. Incubation of the other 6-arylpteridinones **1b** and **1c** with the enzyme extract showed that these 6-aryl derivatives too were easily oxidized into the corresponding lumazines **13b** and **13c**. From the 7-arylpteridinones **2a-c** only **2a** was oxidized at a measurable rate. After incubation overnight at room temperature only **2b** did show a slow change in the UV-spectrum.

Table 2.2 Ultraviolet spectra for 6-, 7-aryl-4(3H)-pteridinones and 6-aryl-4(3H)-pteridinone-8-oxides [λin nm (log ε)] [a].

1a	pH = 5 [b]	229 (4.10)		283 (4.19)	345 (4.02)
	8 [c]	237 (4.08)		274 (4.29)	352 (3.99)
	11 [d]	243 (4.16)		273 (4.32)	361 (4.00)
1b	5	228 (4.11)		288 (4.27)	353 (4.06)
	8	241 (4.11)		282 (4.31)	360 (4.03)
	11	246 (4.22)		280 (4.34)	366 (4.04)
1c	5	227 (4.17)		297 (4.31)	365 (4.09)
	8	243 (4.13)		292 (4.32)	368 (4.07)
	11	248 (4.23)		291 (4.33)	373 (4.06)
2a	5	220 (4.18)	250 (4.23)		338 (4.19)
	8	224 (4.20)	250 (4.18)	263 (4.12)sh[e]	345 (4.14)
	11	230 (4.22)	250 (4.12)	267 (4.15)	352 (4.08)
2b	5	224 (4.26)	255 (4.25)		347 (4.28)
	8	228 (4.28)	256 (4.25)		351 (4.27)
	11	234 (4.30)	260 (4.22)	323 (4.00)sh	356 (4.23)
2c	5	224 (4.23)	261 (4.20)		362 (4.35)
	8	233 (4.26)	263 (4.18)		361 (4.33)
	11	239 (4.32)	270 (4.20)		364 (4.30)
11a	5	222 (4.19)		295 (4.46)	363 (3.90)
	8	227 (4.23)	251 (4.06)	295 (4.45)	380 (4.01)
11b	5	224 (4.12)		302 (4.46)	368 (3.91)
	8	229 (4.25)	255 (4.08)	300 (4.46)	383 (3.98)
11c	5	227 (4.10)	288 (4.19)sh	314 (4.44)	380 (3.89)
	8	232 (4.24)	272 (4.10)sh	307 (4.46)	391 (3.99)

[a] Each compound was dissolved in the minimal amount of 0.01 M potassium hydroxide and from this stock solution (40 mg/100 ml) the final dilution was made in the appropriate buffer.

[b] Sodium acetate buffer.

[c] Tris-HCl buffer.

[d] Potassium phosphate buffer.

[e] sh = shoulder.

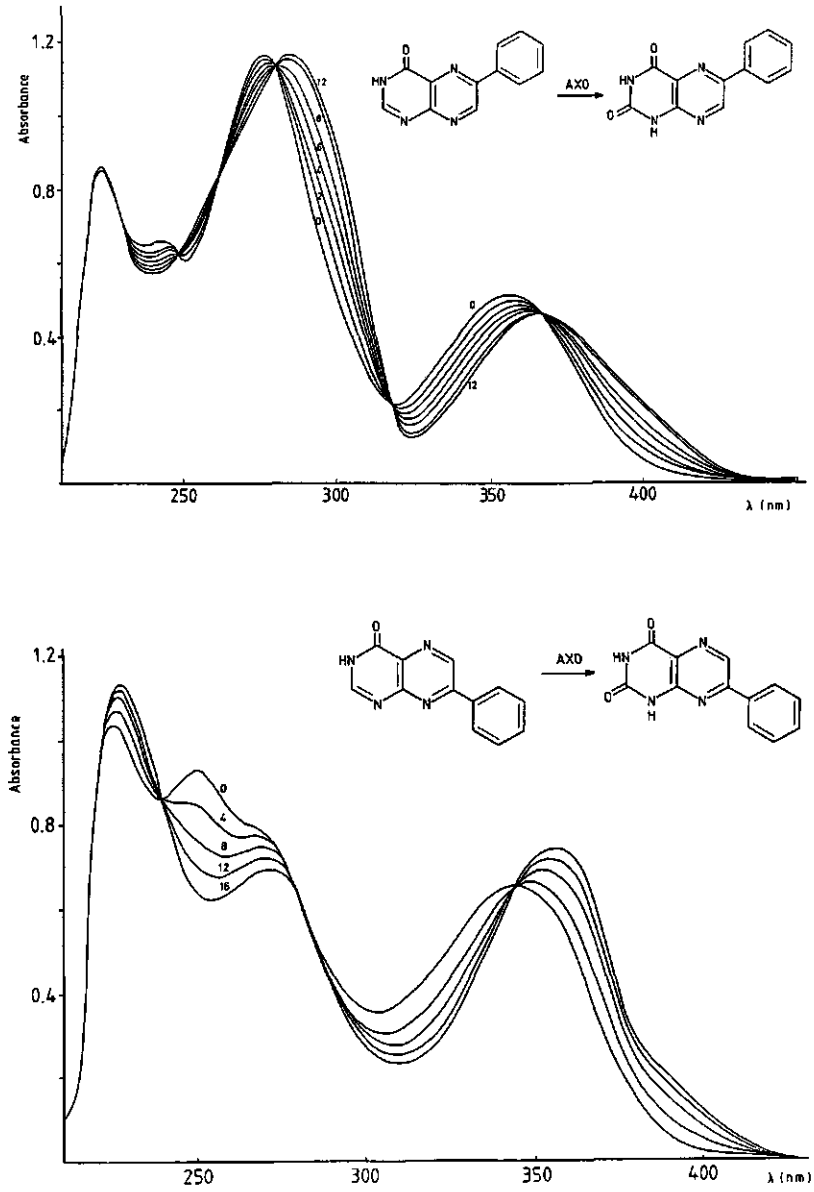


Figure 2.1 UV-spectra, as recorded for 5.10^{-5} M solutions of **1a** *and* **2a** *after the addition of 190 µg of AXO. Spectra were periodically scanned during the reaction (the time in minutes is indicated by the numbers above the lines).*

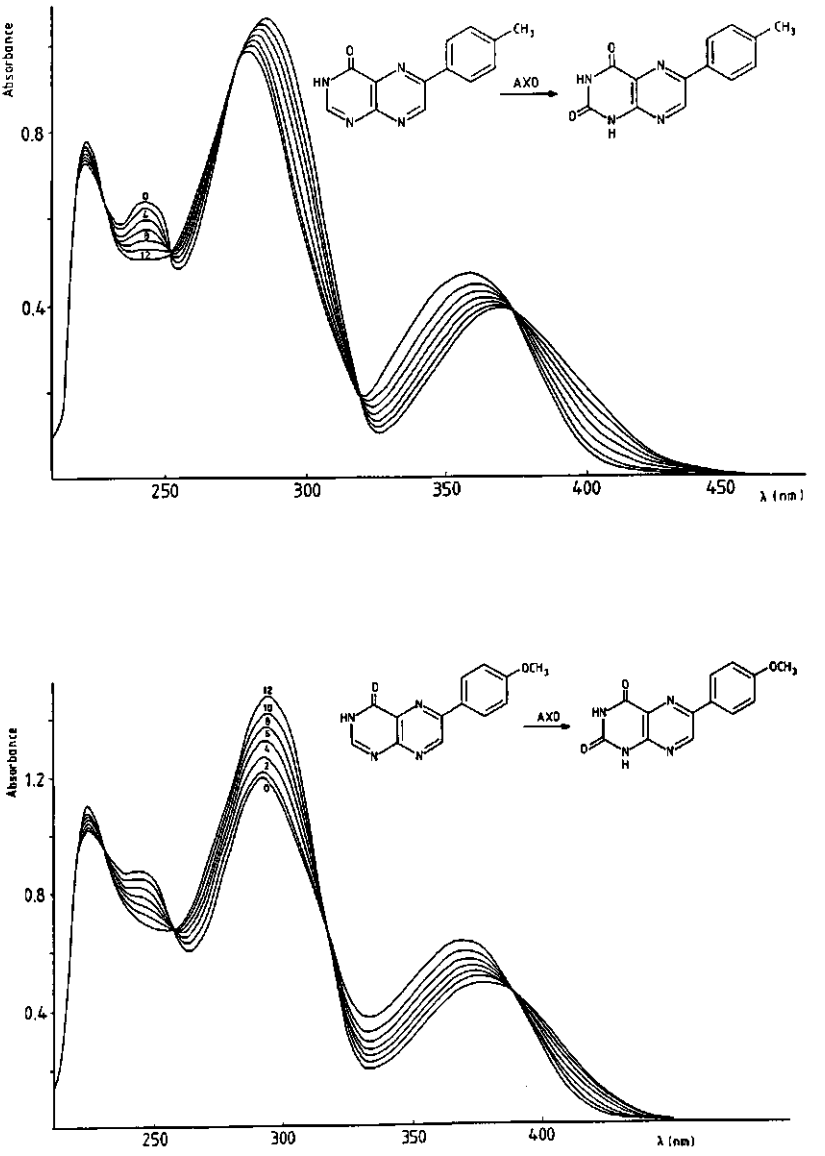
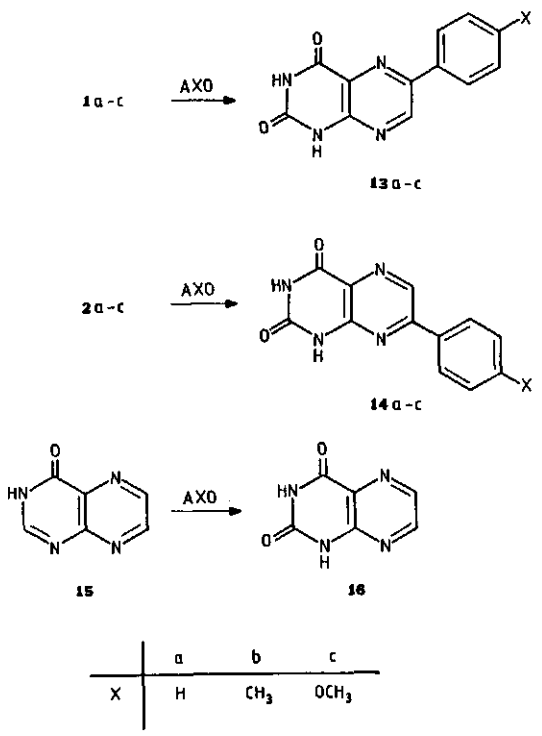


Figure 2.2 UV-spectra, as recorded for 5.10^{-5} M solutions of **1b** *and* **1c** *after the addition of 190 µg of AXO. Spectra were periodically scanned during the reaction (the time in minutes is indicated by the numbers above the lines).*



Scheme 2.4

Since the enzymatic oxidation resulted in the formation of only one product and not in a mixture of products we investigated whether these lumazines could be prepared on a small laboratory scale (50 to 70 mg) using immobilized *Arthrobacter* M-4 cells. For this purpose the cells were immobilized in gelatine crosslinked with glutaraldehyde by the method previously described [7]. In order to enhance the solubility of the substrates **1a-c**, the enzymatic oxidation of these compounds with immobilized *Arthrobacter* cells was carried out at pH=8.0, although the optimal pH for this bacterial enzyme is around 7.2. Oxidation for about one week at room temperature resulted in the complete conversion of **1** into **13**. The structures of the products were identified by comparison of the nmr data, UV-data and mass spectrometric data with those of an authentic specimen (Tables 2.3 and 2.4).

From the 7-arylpteridinones **2a-c** only **2a** was rather quickly converted, in contrast to its *p*-methylphenyl and *p*-methoxyphenyl derivatives **2b** and **2c**. However, after five days of incubation with immobilized cells, **2b** gave 70% of oxidized product, whereas it took **2c** three weeks of incubation at room temperature with immobilized cells to give only 15% of the corresponding lumazine

Table 2.3 *^{13}C nmr data of 6- and 7-aryl-2,4(1H,3H)-pteridinediones **13a-e** and **14a-e***

Compound	C-2	C-4	C-6	C-7	C-9	C-10
16	149.9	160.9	140.2	148.2	149.6	128.0
13a	150.0	161.2	148.5	145.7	147.1	126.9
13b	149.8	161.0	148.2	145.3	147.2	126.7
13c	150.0	161.3	147.9	145.3	147.2	126.7
14a	150.2[a]	161.0	137.4	154.3	149.1[a]	126.4
14b	150.3[a]	161.0	137.2	154.3	149.1[a]	126.0
14c	150.3[a]	161.1	137.0	154.1	149.1[a]	125.4

[a] These signals may be interchanged.

14c as deduced from ^{1}H nmr spectroscopy.

The enzymatic conversions of **1a-c** and **2a** were all quantitative but some losses occurred during the isolation procedure. The yields of the isolated products varied from 86 to 96%. Table 2.4 summarizes the yields and analytical data of the lumazines obtained in the reactions with immobilized cells.

The kinetic constants Vm and Km were determined for the AXO-mediated reaction of **1a** and **2a** at pH=8.0 assuming that the reaction follows simple Michaelis-Menten kinetics. For reasons of comparison we also determined the kinetic parameters of 4(3*H*)-pteridinone (**15**). This compound is exclusively oxidized at the C-2 position, since the UV-spectrum after the oxidation of **15** is identical to that of lumazine (**16**). Lumazine is not further oxidized by the bacterial xanthine oxidase. This is in contrast to the behaviour of **15** towards bovine milk xanthine oxidase, which resulted in the formation of 2,4,7(1*H*,3*H*,8*H*)-pteridinetrione via 4,7(3*H*,8*H*)-pteridinedione [31b]. For this reason the comparison between **1a**, **2a** and **15** is completely justified, since oxidation takes place at the same carbon atom. For the 6-phenyl derivative **1a** the maximum rate of oxidation at C-2 is about two times higher than that at C-2 in the 7-phenylpteridinone **2a**. The Michaelis constant Km of the compounds **1a** and **2a** are about ten times lower than the corresponding values found for 4(3*H*)-pteridinone and xanthine [33]. The position of the phenyl group in the pteridine is apparently important for adequate fitting of the substrate in the active site of the enzyme. In Table 2.5 also the Vm/Km values are listed. These figures reflect the relative efficiency of enzymatic oxidation for each of

Table 4.2 Yields and analytical data of products obtained using immobilized Arthrobacter *M-4 cells.*

Substrate	Product	Yield	^{1}H nmr data [δ, dmso-d_6]	Ultraviolet Spectra [λ (log ε)]					Formula	Exact mass	
										Exp.	Theor.
1a	13a	92%	7.50-7.80 (m,3H,ArH), 8.05-8.25 (m,2,ArH),	pH=5[a]		273 (4.36)		354 (4.01)			
			9.23 (s,1H,H-7), 11.70 (br s,1H,NH), 11.92	8[b]		286 (4.30)		364 (3.92)	$C_{12}H_8N_4O_2$	240,0648	240,0647
			(br s,1H,NH).	13[c]		280 (4.39)		384 (3.98)			
1b	13b	87%	2.43 (s,3H,CH_3), 7.66 (d, J=7.5 Hz,2H,ArH),	5		279 (4.40)		361(4.00)			
			8.05 (d,J=7.5 Hz,2H,ArH), 9.27 (s,1H,H-7),	8		289 (4.36)		369 (3.93)	$C_{13}H_{10}N_4O_2$	254,0806	254,0804
			11.80 (br s,2H,NH).	13		286 (4.38)		388 (3.98)			
1c	13c	86%	3.87 (s,3H,OCH_3), 7.10 (d,J=9 Hz,2H,ArH),	5		288 (4.40)		376 (3.93)			
			8.08 (d, J=9 Hz,2H,ArH), 9.20 (s,1H,H-7),	8		294 (4.42)		375 (3.92)	$C_{13}H_{10}N_4O_3$	270,0754	270,0753
			11.65 (br s,1H,NH), 11.90 (br s, 1H, NH).	13		298 (4.46)		384 (3.96)			
2a	14a	96%	7.50-7.73 (m,3H,ArH), 8.10-8.36 (m,2H,ArH),	5	223 (4.33)	260 (3.98)sh[d]		349 (4.33)			
			9.17 (s,1H,H-6), 11.63 (s,1H,NH), 11.95 (br	8	226 (4.33)	271 (4.11)		353 (4.19)	$C_{12}H_8N_4O_2$	240,0649	240,0647
			s,1H,NH).	13	230 (4.35)	268 (4.32)		372 (4.07)			
2b	14b	70%[e]	2.48 (s,3H,CH_3), 7.43 (d,J=7.5 Hz,2H,ArH),	5	230 (4.29)	256 (3.89)sh	280 (3.81)sh	355 (4.38)			
			8.15 (d,J=7.5 Hz,2H,ArH), 9.12 (s,1H,H-6),	8	231 (4.23)	274 (4.02)		358 (4.26)	$C_{13}H_{10}N_4O_2$	254,0801	254,0804
			11.65 (br s,1H,NH), 11.92 (br s,1H,NH).	13	236 (4.32)	270 (4.24)	300 (3.93)sh	372 (4.12)			
2c	14c	15%[e]	3.92 (s,3H,OCH_3), 7.29 (d,J=9 Hz,2H,ArH),	5	235 (4.22)	268 (3.80)sh	300 (3.73)	367 (4.39)			
			8.33 (d,J=9 Hz,ArH), 9.15 (s,1H,H-7), 11.63	8	235 (4.23)	272 (3.94)sh	304 (3.81)sh	369 (4.30)	$C_{13}H_{10}N_4O_3$	270,0755	270,0753
			(br s,1H,NH), 11.88 (br s,1H,NH).	13	244 (4.27)	270 (4.12)sh	313 (3.90)sh	378 (4.17)			

[a] Sodium acetate buffer.

[b] Tris-HCl buffer.

[c] 0.1 M potassium hydroxide.

[d] sh= shoulder.

[e] Deduced from 300 MHz ^{1}H nmr spectrum taken after isolation of the crude reaction mixture obtained after separation of the immobilized cells.

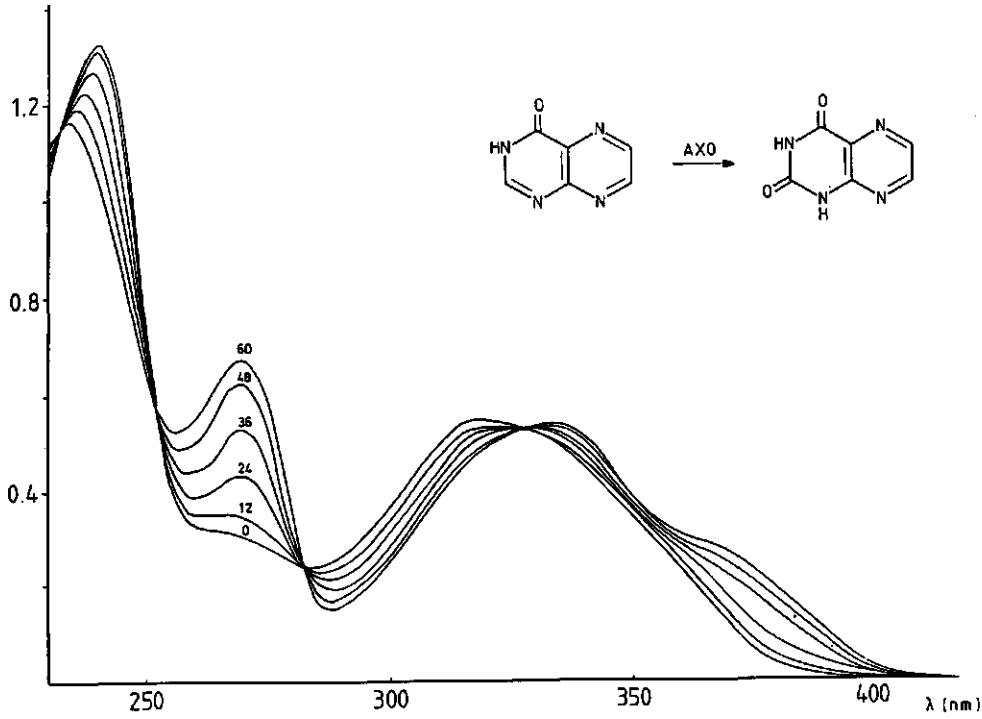


*Figure 2.3 UV-spectra, as recorded for 8.10^{-5} M solution of 4(3H)-pteridinone (**15**) after the addition of 190 µg of AXO. The spectra were taken every twelve minutes. After one hour the reaction was complete.*

*Table 2.5 Kinetic parameters for the oxidation of **1a**, **2a** and **15** by the crude cell-free extract at pH=8.0* [a].

	Km [b]	Vm [c]	Vm/Km [d]
1a	11.8 ± 0.9	0.092 ± 0.005	7.8
2a	9.5 ± 0.8	0.042 ± 0.003	4.4
15	109.9 ± 9.7	0.102 ± 0.008	0.93

[a] The oxidation rate for 1-methylxanthine with the bacterial xanthine oxidase preparations used here was 0.25 µmol ± 0.03 µmol/min.mg.

[b] In µmol/l.

[c] In µmol/min.mg.

[d] In ml/min.mg.

these substrates. Even then, the increase in relative efficiency is largely due to the presence of the phenyl group in both **1a** and **2a** and point to the existence of a hydrophobic regio in the vicinity of the active center of the bacterial enzyme; this proposal parallels that of Baker et al. [34] for bovine milk xanthine oxidase.

2.4 EXPERIMENTAL SECTION

Melting points were determined on a Kofler hot stage equipped with a microscope and a polarizer and they are uncorrected. ^{1}H nmr spectra were measured using an Hitachi Perkin-Elmer R-248 or a Varian EM 390 spectrometer, with TMS as internal standard. ^{13}C nmr spectra were recorded using dimethylsulfoxide-d_6 as solvent and internal standard on a Bruker CXP 300 spectrometer equipped with a B-VT 1000 variable temperature controller. Infrared spectra were obtained with a Perkin Elmer 237 or an Hitachi EPI-G3. Mass spectra were recorded on a AEI MS 902 instrument. Field desorption mass spectra were taken of all the compounds with an N-oxide and a mp. higher than 200°, most of the time the molecular ion was observed with a small intensity for $(M-16)^{+}$, unlike the spectra taken with the electron impact technique, where deoxygenation of these compounds occurred. Ultra violet spectra were determined using a Beckmann DU-7, Aminco DW-2A or Varian DMS 100 spectrophotometer. Column chromatography was carried out on Merck Silica gel 60 (70-230 mesh ASTM) and Silical GF from Merck was used for analytical thin layer chromatography, using the following solvent systems: A, chloroform-ethanol (95:5); B, chloroform-methanol (9:1); C, dichloromethane-methanol (9:1); D, benzene-ethyl acetate (7:3).

Preparation of starting materials and reference compounds

2-Amino-2-cyanoaceetamide (**6a**) [25,26], benzyl 2-amino-2-cyanoacetate methanesulfonic acid salt (**6c**) [24], 2-amino-3-ethoxycarbonyl-5-phenylpyrazine-1-oxide (**7a**) [17], 6-phenyl-2,4(1*H*,3*H*)-pteridinedione (**13a**) [30], 6-(*p*-methylphenyl)-2,4(1*H*,3*H*)-pteridinedione (**13b**) [30], 6-(*p*-methoxyphenyl)-2,4(1*H*,3*H*)-pteridinedione (**13c**) [30], 4(3*H*)-pteridinone (**15**) [35] and 2,4(1*H*,3*H*)-pteridinedione (**16**) [35,36] were prepared according to the prescriptions given in the literature.

Ethyl 2-amino-2-cyanoacetate p-*toluenesulfonic acid salt (6b)* [37,38]

This compound was synthesized using a modification [25,26] of previously

described methods which were adapted for preparation on a large scale. Under nitrogen ethyl 2-oximino-2-cyanoacetate [39] (60 g, 0.42 mol, mp. 133°) was covered with 360 ml of water. The suspension was carefully treated with 180 ml of a saturated sodium bicarbonate solution. To the yellow solution sodium dithionite (90%, 227 g, 1.17 mol) was added in portions over a period of about an hour. The temperature rose about 20° during this time, but was kept below 35° to avoid lower yields or an impure product. After the addition, stirring was continued for 30 minutes. Extraction with five portions of distilled chloroform (5x300 ml), drying of the extracts over magnesium sulfate and evaporation *in vacuo* (below 35°) yielded 32-36 g of an oil (59-66%). This oil was immediately diluted with 300 ml of dry ether. To this solution *p*-toluenesulfonic acid monohydrate (60 g, 0.32 mol) in 150 ml of ethanol was added. Under vigourous stirring the solution was slowly diluted with dry ether to 1000 ml to induce crystallization. The mixture was kept overnight at -20°. After filtering, washing with cold dry ether and drying over phosphorus pentoxide, 65-70 g of a snow-white precipitate was obtained (48-52%), mp. 118-120° (lit. [38] mp. 125-6°, lit. [37] mp. 115-7°); it was used without further purification in the cyclization reactions.

*Arylglyoxal 2-oximes (**5a-c**)*

Selenium dioxide (37 g, 0.33 mol) was mixed with 10 ml of water and 250 ml of dioxane at 50-60°. When almost all selenium dioxide had gone into solution 0.3 mol of **3** was added in one portion. After refluxing for 16 hours the solution was filtered hot to remove elemental selenium and the yellow-orange filtrate was concentrated *in vacuo*. The resulting reddish yellow oil was diluted with 300 ml of chloroform and washed quickly with a saturated sodium bicarbonate solution (100 ml) and water (100 ml). After drying over magnesium sulfate, any precipitate formed during this time was removed by filtration through Celite. The yellow coloured solution turned reddish-yellow due to formation of spots of amorphous red selenium. After evaporation of the chloroform, the resulting oil was purified by destillation *in vacuo* (**4a**: 70-72%, bp 90-95° at 20 mm Hg, lit. [20b] bp 95-97° at 25 mm Hg; **4b**: 81-83%, bp 76-80° at 3 mm Hg, lit. [40] bp 104-110° at 10 mm Hg; **4c**: 69-72%, bp 145-147° at 13 mm Hg, lit.[40] bp 105-110° at 3 mm Hg). The resulting yellow oil (for **4c**: the oil solidified giving a product with mp 100-104°) was converted into the hydrate by refluxing in 400 ml of water (800 ml for **4c**) during 3 hours. Charcoal was added and after keeping at reflux for ten minutes the solution was filtered. After standing overnight at 4° the white precipitate was isolated by filtration.

After washing with ice-water the hydrate [41] was dried over phosphorus pentoxide (hydrate of **4a**: 80%, mp 70-74°, lit. [20b] mp 71°, lit. [42] mp 76-77°; hydrate of **4b**: 75%, mp 90-94°, then mp 105-108°, lit. [42] mp 98-99°; hydrate of **4c**: 72%, mp 75-80°, then mp 110-114°, lit. [42] 126-128°, lit. [43a] 89-92°, lit. [44] mp 84°). When the product is not converted into the hydrate, di- and polymerization is likely to occur, even when the arylglyoxal is kept at -30° (as indicated by nmr spectroscopy). For conversion into **5a-c** also freshly distilled **4a-c** can be used.

Method A. Freshly distilled **4c** (5 g, 30.5 mmol) was dissolved in 20 ml of methanol and 80 ml of water. After addition of acetonoxime (2.7 g, 37 mmol) the pH was brought to 4 with 2N aqueous hydrochloric acid and heated for two hours at 50°. During this time a white-yellow precipitate was gradually formed. After cooling in an ice-bath the product was filtered off and washed well with ice-water, and dried over phosphorus pentoxide. When the temperature was raised to reflux, the acid hydrolysis of acetonoxime to hydroxylamine became competitive with the transoximation resulting in a mixture of dioxime [22] and **5**.
Compounds **5a** and **5b** were prepared using the same procedure as described above starting from 5 g of **5a** or **5b** in 10 ml of methanol and 40 ml of water.

Method B. Acetonoxime (0.85 g, 11.5 mmol), 10 mmol of the hydrate of **4a-c** and 20 ml of water was brought to pH=4 with 2 N aqueous hydrochloric acid. After heating and stirring for two hours at 50° the solution was cooled.

Work-up procedure was the same as described in method A. Stirring overnight at room temperature gave about the same results, but at 50° the reaction proceeded quicker. The products prepared according to both methods were pure enough for further reactions. Recrystallization was accomplished from chloroform (**5a**) and methanol-water (**5b** and **5c**).

*Phenylglyoxal-2-oxime (phenylglyoxalaldoxime) (**5a**)*

Yield 82% (method A), 91% (method B), mp 127-8° (lit. [45] mp 126-7°).

*(p-Methylphenyl)glyoxal-2-oxime (**5b**)*

Yield 80% (method A), 93% (method B), mp 98-100° (lit. [46] mp 100°).

*(p-Methoxyphenyl)glyoxal-2-oxime (**5c**)*

Yield 86% (method A), 92% (method B), mp 117-118° (lit. [21b] mp 119°).

*2-Amino-3-ethoxycarbonyl-5-(p-methylphenyl)pyrazine-1-oxide (**7b**)*

In 10 ml of anhydrous methanol a mixture of compound **5b** (1.7 g, 10.4 mmol)

and **6b** (3.2 g, 10 mmol) was stirred at 35° for five days. After cooling in ice-water for thirty minutes the yellow precipitate was filtered, washed with ice-cold methanol (10 ml) and cold ether (2 x 30 ml) to give 1.83 g (67%) of a bright yellow solid. Work-up after ten days of stirring gave a yield of 83%. Concentrating of the filtrate and extraction with ethyl acetate and chloroform [17] yielded an additional amount of 0.15-0.20 g of **7b**. Recrystallization from 2-propanol gave bright yellow needles of **7b** with mp 173-175°; ^{1}H nmr (90 MHz, deuteriochloroform): δ 1.48 (t, J=7.1 Hz, 3H, CH_3), 2.41 (s, 3H, CH_3), 4.48 (q, J=7.1 Hz, 2H, CH_2), 7.33 (d, J=7.5 Hz, 2H, ArH), 7.35 (br s, 2H, NH_2), 7.76 (d, J=7.5 Hz, 2H, ArH), 8.63 (s, 1H, H-6).

Anal. Calcd for $C_{14}H_{15}N_3O_3$ (273.28): C, 61.53; H, 5.53. Found: C 61.33; H, 5.32.

*2-Amino-3-ethoxycarbonyl-5-(*p*-methoxyphenyl)pyrazine-1-oxide (**7c**)*

This compound was prepared similar to the method described above for **7b**, starting from **5c** (1.80 g, 10.1 mmol) and **6b** (3.2 g, 10 mmol) in 10 ml of absolute methanol. Stirring for five days yielded 2.15 g (74%) of a yellow solid. Recrystallization from 2-propanol afforded bright yellow, fluffy needles of **7c**, mp 170-172°; ^{1}H nmr (90 MHz, deuteriochloroform): δ 1.49 (t, J=7.5 Hz, 3H, CH_3), 3.88 (s, 3H, OCH_3), 4.50 (q, J=7.5 Hz, 2H, CH_2), 6.98 (d, J=9 Hz, 2H, ArH), 7.30 (br s, 2H, NH_2), 7.81 (d, J=9 Hz, 2H, ArH), 8.57 (s, 1H, H-6)

Anal. Calcd for $C_{14}H_{15}N_3O_4$ (289.28): C, 58.12; H, 5.23. Found: C, 58.24; H, 4.94.

*2-Amino-3-benzyloxycarbonyl-5-phenylpyrazine-1-oxide (**8a**)*

A mixture of **5a** (0.75 g, 5 mmol) and **6c** (1.43 g, 5 mmol) was stirred in 10 ml of absolute methanol. After five minutes a clear yellow solution was obtained, from which after fifteen minutes a yellow precipitate started to separate. Stirring was continued and after five days the reaction mixture was diluted with 50 ml of ice water. A copious precipitate was formed. After cooling for one hour in the refrigerator the precipitate was isolated by filtration, washed with water (20 ml), cold methanol (5 ml) and finally with cold ether (2 x 20 ml) to give 1.45 g (90%) of a yellow precipitate; recrystallization from 1-propanol gave bright yellow, fluffy needles of **8a**, mp 164-166°; ^{1}H nmr (90 MHz, deuteriochloroform): δ 5.50 (s, 2H, CH_2), 7.22-7.60 (m, 10H, ArH, Ar'H, NH_2); 7.80-7.95 (m, 2H, ArH), 8.65 (s, 1H, H-6).

Anal. Calcd for $C_{18}H_{15}N_3O_3$ (321.32): C, 67.28; H, 4.70. Found: C, 67.19; H, 4.52.

*2-Amino-3-benzyloxycarbonyl-5-(p-methylphenyl)pyrazine-1-oxide (**8b**)*

This compound was prepared according to the method described for **8a**, starting from **5b** (1.80 g, 10.9 mmol) and **6c** (2.86 g, 10 mmol) in 15 ml of anhydrous methanol. Stirring for five days produced 2.75 g (81%) of a golden yellow solid. Filtration and recrystallization from 1-propanol afforded bright yellow, fluffy needles of **8b**, mp. 184-6°; ^{1}H nmr (deuteriochloroform + dmso-d_6): δ 2.37 (s, 3H, CH_3), 5.47 (s, 2H, CH_2), 7.23 (d, J=7.5 Hz, 2H, ArH), 7.35-7.56 (m, 5H, ArH), 7.65 (br s, 2H, NH_2, D_2O exchangeable), 7.83 (d, J=7.5 Hz, 2H, ArH), 8.90 (s, 1H, H-6).

Anal. Calcd for $C_{19}H_{17}N_3O_3$ (335.35): C, 68.05; H, 5.11. Found: C, 68.06; H, 4.95.

*2-Amino-3-benzyloxycarbonyl-5-(p-methoxyphenyl)pyrazine-1-oxide (**8c**)*

This compound was synthesized according to the same procedure as given for **8a**, using **5c** (2.01 g, 11.2 mmol) and **6c** (2.86 g, 10 mmol) in 15 ml of anhydrous methanol. A bright yellow solid (2.58 g, 76%) was obtained after five days of stirring. Filtration and recrystallization from 1-propanol yielded bright yellow, fluffy needles of **8c**, mp. 151-3°; ^{1}H nmr (deuteriochloroform): δ 3.87 (s, 3H, OCH_3), 5.50 (s, 2H, CH_2), 6.98 (d, J=9 Hz, 2H, ArH), 7.25 (s, 2H, NH_2), 7.30-7.60 (m, 5H, ArH), 7.82 (d, J=9 Hz, 2H, ArH), 8.63 (s, 1H, H-6).

Anal. Calcd for $C_{19}H_{17}N_3O_4$ (351.35): C, 64.95; H, 4.88. Found: C, 64.82; H, 4.74.

*Preparation of 2-amino-3-carbamoyl-5-arylpyrazine-1-oxides (**9a-c**)*

These compounds were prepared by three methods indicated by A, B or C. The yields of compounds **9a-c** obtained by these methods are summarized in Table 2.6.

Method A. A solution of 2-amino-2-cyanoaceetamide **6a** (3.0 g, 30.3 mmol) in 10 ml of glacial acetic acid was mixed with 10 mmol of **5a-c** in 15 ml of glacial acetic acid [17b]. Stirring was continued at room temperature for seven days. The slurry was diluted with 80 ml of water, the product was filtered and washed with water (30 ml), ethanol (20 ml) and cold ether (50 ml) and dried over phosphorus pentoxide. The product was recrystallized from dimethylformamide.

Method B. A suspension of 2 mmol of **7a-c** in 100 ml of dry liquid ammonia was stirred under reflux at -33° during three hours [27]; all starting material was then converted into the amide **9a-c** as indicated by tlc (solvent B or C). After evaporation of most of the liquid ammonia overnight, 30 ml of 1-propanol was added to the residue and the residue was collected by filtration. The same procedure was applied for the benzylesters **8b** and **8c** (2 mmol of each in 200 ml

of dry liquid ammonia).

Method C. A solution of 4 mmol of **7a**, **8b**, or **8c** in 200 ml of dry 1-propanol saturated with dry ammonia at 0° was stirred at room temperature. After a few hours a bright yellow precipitate appeared. The conversion to **9a-c** was complete after 30 to 36 hours (tlc, solvent B or C). The precipitate was collected; concentration of the filtrate to a volume of 20 ml yielded a second crop. Total yield is given in Table 2.6. Using aqueous ammonia instead of propanolic ammonia resulted in a very slow conversion to the amide and subsequent hydrolysis to the corresponding acid.

*Table 2.6 Yields of 2-amino-3-aminocarbonyl-5-arylpyrazine-1-oxides **9a-c** from the compounds **5**, **7**, **8** by the methods A, B and C.*

Compound	Method A	Method B		Method C	
	from **5**	from **7**	**8**	from **7**	**8**
9a	64%			98%	
9b	81%	89%	94%		96%
9c	52%	96%	95%		96%

*2-Amino-3-carbamoyl-5-phenylpyrazine-1-oxide (**9a**)*

Bright yellow needles, mp 281-3° (mp [17b] 280-2°); ^{1}H nmr (90 MHz, dmso-d_6): δ 7.40-7.62 (m, 3H, ArH), 7.91 (br s, 2H, NH_2), 8.10-8.27 (m, 2H, ArH), 7.91 and 8.40 (br s, 2H, $CONH_2$), 9.08 (br s, 1H, H-6); ms: m/e 230 (M^+, 100), 214 (M^+-16, 27).

Anal. Calcd for $C_{11}H_{10}N_4O_2$ (230.22) : C, 57.38; H, 4.38. Found : C, 57.25; H, 4.20.

*2-Amino-3-carbamoyl-5-(p-methylphenyl)pyrazine-1-oxide (**9b**)*

Bright yellow fine needles, mp 274-6°; ^{1}H nmr (90 MHz, dmso-d_6): δ 2.33 (s, 3H, CH_3), 7.24 (d, J=7.5 Hz, 2H, ArH), 7.88 (br s, 2H, NH_2), 8.07 (d, J=7.5 Hz, 2H, ArH), 7.88 and 8.39 (br s, 2H, $CONH_2$), 9.03 (s, 1H, H-6); ms : m/e 244 (M^+, 100), 228 (M^+-16, 79).

Anal. Calcd for $C_{12}H_{12}N_4O_2$ (244.25) : C, 59.00; H, 4.95. Found : 58.98; H, 4.83.

*2-Amino-3-carbamoyl-5-(p-methoxyphenyl)pyrazine-1-oxide (**9c**)*

Long bright yellow needles, mp 254-6°; ^{1}H nmr (90 MHz, dmso-d_6) : δ 3.80

(s, 3H, OCH_3), 6.98 (d, J=9 Hz, 2H, ArH), 7.82 (br s, 2H, NH_2), 8.12 (d, J=9 Hz, 2H, ArH), 7.82 and 8.40 (br s, 2H, $CONH_2$), 9.01 (s, 1H, H-6); ms : m/e 260 (M^+, 100) 244 (M^+-16, 59).

Anal. Calcd for $C_{12}H_{12}N_4O_3$ (260.25): C, 55.38; H, 4.65. Found: C, 55.68; H, 4.50.

*Preparation of 2-amino-3-carbamoyl-5-arylpyrazines (**10a-e**)*

Method A. Reduction of **9** with sodium dithionite. A suspension of 3 mmol of **9a-c** in 15 ml of boiling water was treated with sodium dithionite (90%, 6.0 g, 31 mmol) in small portions over a period of 15 minutes. The mixture was kept at reflux for 24 hours. Monitoring by tlc (solvent B or C) revealed that at least 18-21 hours were necessary for complete deoxygenation. After cooling, the pale yellow solid was filtered and washed with 20 ml of water, 30 ml of cold methanol and finally with cold ether. Analytical samples were prepared by recrystallization from 1-propanol.

Method B. Reduction of **9** with phosphorus trichloride. To a solution of 2 mmol of **9a-c** in 200 ml of dry THF at 0° was added dropwise 2 ml (3.14 g, 22.8 mmol) of phosphorus trichloride. The mixture was stirred for 2 hours at room temperature, evaporated to a volume of 20-30 ml, and the residue was carefully diluted with ice water to a volume of about 100 ml. The precipitated yellow solid was collected by filtration and dried by suction. Analytical samples were prepared by sublimation *in vacuo* at 200° (1 mm Hg).

*2-Amino-3-carbamoyl-5-phenylpyrazine (**10a**)*

Yield 97% (method A), 89% (method B); pale yellow, short needles, mp 239-41° (lit [47] mp 239-40°); ^1H nmr (90 MHz, dmso-d_6) : δ 7.30-7.51 (m, 3H, ArH), 7.63 (br s, 2H, NH_2), 8.05-8.20 (m, 2H, ArH), 7.63 and 8.15 (br s, 2H, $CONH_2$), 8.81 (br s, 1H, H-6); ms : m/e 214 (M^+).

Anal. Calcd for $C_{11}H_{10}N_4O$ (214.22): C, 61.67; H, 4.70. Found: C, 61.50; H, 4.48.

*2-Amino-3-carbamoyl-5-(p-methylphenyl)pyrazine (**10b**)*

Yield 89% (method A), 81% (method B); pale yellow, short needles, mp 248-50°, ^1H nmr (90 MHz, dmso-d_6) : δ 2.37 (s, 3H, CH_3), 7.23 (d, J=7.5 Hz, 2H, ArH), 7.55 (br s, 2H, NH_2), 8.05 (d, J=7.5 Hz, 2H, ArH), 7.55 and 8.22 (br s, 2H, $CONH_2$), 8.80 (s, 1H, H-6); ms : m/e 228 (M^+).

Anal. Calcd for $C_{12}H_{12}N_4O$ (228.25):C, 63.14; H, 5.30. Found: C, 62.87; H, 5.16.

*2-Amino-3-carbamoyl-5-(p-methoxyphenyl)pyrazine (**10c**)*

Yield 86% (method A), 83% (method B); dark yellow, long needles, mp. 210-2°, ^{1}H nmr (90 MHz, dmso-d_6): δ 3.80 (s, 3H, OCH_3), 6.98 (d, J=9 Hz, 2H, ArH), 7.56 (br s, 2H, NH_2), 8.08 (d, J=9 Hz, 2H, ArH), 7.56 and 8.24 (br s, 2H, $CONH_2$), 8.76 (s, 1H, H-6); 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, 59.00; H, 4.89.

*Preparation of 6-aryl-4(3H)-pteridinone-8-oxides (**11a-c**)*

Method A. A solution of 5 ml of triethyl orthoformate and 10 ml of dimethylaceetamide containing 0.3 g of **9a-c** was heated and stirred at 140° (oil bath) during 12 hours (16 hours for **9c**). After cooling, the precipitate was collected, washed with water, methanol and ether and dried over phosphorus pentoxide at 100°. Heating at 160° instead of 140° resulted in a brownish solution from which after cooling no precipitation of product occurred [17b].

Method B. A mixture of 0.6 g of **9a-c**, 6 ml of triethyl orthoformate and 10 ml of dimethylformamide was heated with stirring during 12 hours (16 hours for **9c**) at 160° (oil bath). Work-up was the same as described above for method A. The compounds are recrystallized from dimethylsulfoxide.

*6-Phenyl-4(3H)-pteridinone-8-oxide (**11a**)*

Yield 47% (method A), 55% (method B); cream coloured needles, mp. 325° dec. (lit. [17] > 320°); ^{1}H nmr (90 MHz, dmso-d_6): δ 7.43-7.60 (m, 3H, ArH), 8.06-8.20 (m, 2H, ArH), 8.11 (s, 1H, H-2), 9.09 (s,1H, H-7); field desorption ms: m/e 240 (M^+).

Anal. Calcd for $C_{12}H_8N_4O_2 \cdot \frac{1}{2}H_2O$ (249.23): C, 57.83; H, 3.64. Found: C, 57.60, H, 3.88.

*6-(p-Methylphenyl)-4(3H)-pteridinone-8-oxide (**11b**)*

Yield 57% (method A), 67% (method B); white needles, mp. 322-4° dec.; ^{1}H nmr (90 MHz, dmso-d_6): δ 2.38 (s, 3H, CH_3), 7.35 (d, J=7.5 Hz, 2H, ArH), 8.07 (d, J=7.5 Hz, 2H, ArH), 8.24 (s, 1H, H-2), 9.30 (s, 1H, H-7); field desorption ms: m/e 254 (M^+).

Anal. Calcd for $C_{13}H_{10}N_4O_2$ (254.24): C, 61.41; H, 3.96. Found: C, 61.49, H, 3.80.

*6-(p-Methoxyphenyl)-4(3H)-pteridinone-8-oxide (**11c**)*

Yield 63% (method A), 74% (method B); pale yellow crystals, mp. 325-8°

dec; ^{1}H nmr (90 MHz, dmso-d_6): δ 3.83 (s, 3H, OCH_3), 7.08 (d, J=9 Hz, 2H, ArH), 8.13 (d, J=9 Hz, 2H, ArH), 8.22 (s, 1H, H-2), 9.27 (s, 1H, H-7); field desorption ms: m/e 270 (M^+).

Anal. Calcd for $C_{13}H_{10}N_4O_3$ (270.24): C, 57.77; H,3.73. Found: C, 57.99; H, 3.95.

*Preparation of 6-aryl-4(3H)-pteridinones (**1a-c**)*

Method A. To a clear yellow solution of 0.14 g of **11a** in 7 ml of 0.5 M sodium hydroxide 0.64 g of 90% sodium dithionite was added and the resulting solution was heated under reflux for fifteen minutes. A precipitate was formed. After cooling and acidification to pH=1-2 with concentrated hydrochloric acid, a yellow brownish precipitate was finally obtained. This was collected by filtration, and redissolved in 5 ml of hot 0.5 N sodium hydroxide. Acidification of the solution gave again a bright yellow precipitate. The mass spectrum of this material revealed that it was a mixture of **1a** and its dihydro and tetrahydro derivative. This mixture was dissolved in 10 ml of water and made alkaline with the minimal amount of 0.1 M sodium hydroxide. After addition of 5 ml of 0.1 M potassium permanganate the solution was stirred for ten minutes at room temperature. To dissolve the brown precipitate of manganese dioxide, sulfur dioxide was bubbled into the mixture for about one minute; a milky white product precipitated. Filtration and washing with water and ethanol gave 0.1 g of **1a** (77% yield). For preparation of **1b** and **1c** a similar procedure was used as described above.

Method B. Compound **10a-c** (150 mg) was heated in a mixture of 5 ml triethyl orthoformate and 5 ml of acetic anhydride at reflux (oil bath, 150°) for two hours. After cooling to room temperature, the dark brown solution was evaporated to dryness. To the residue 5 ml of water and 5 ml of ethanol were added and the solution was stirred for 15 minutes. The precipitate obtained was filtered and washed with water, ethanol and ether and recrystallized from dimethylsulfoxide. Also with sublimation *in vacuo* at 250° (1 mm Hg) analytically pure samples could be obtained.

*6-Phenyl-4(3H)-pteridinone (**1a**)*

Yield 77% (method A), 85% (method B); white short needles, mp. 307-9° (lit. [13], mp. 304° dec); ^{1}H nmr (90 MHz, dmso-d_6): δ 7.50-7.66 (m, 3H, ArH), 8.16-8.30 (m, 2H, ArH), 8.35 (s, 1H, H-2), 9.60 (s, 1H, H-7); ms: m/e: 224 (M^+, 100); 197 (M^+-27, 23), 104 (M^+-120, 58).

Anal. Calcd for $C_{12}H_8N_4O$ (224.25): C, 64.27; H, 3.61. Found: C, 64.54, H, 3.82.

*6-(p-Methylphenyl)-4(3H)-pteridinone (**1b**)*

Yield 45% (method A), 32% (method B); white feathery short needles, mp. 288-90° (lit. [13] mp. 286° dec.); ^{1}H nmr (90 MHz, dmso-d_6): δ 2.38 (s, 3H, CH_3), 7.35 (d, J=7.5 Hz, 2H, ArH), 8.15 (d, J=7.5 Hz, 2H, ArH), 8.33 (s, 1H, H-2), 9.55 (s, 1H, H-7); ms: m/e: 238 (M^+,100), 211 (M^+-27, 14), 118 (M^+-120, 39).

Anal. Calcd for $C_{13}H_{10}N_4O$ (238.24): C, 65.53; H,4.23. Found: C, 65.50; H, 4.07.

*6-(p-Methoxyphenyl)-4(3H)-pteridinone (**1c**)*

Yield 81% (method A), 84% (method B); yellow short needles, mp. 290-2° (lit. [13] mp. 280° dec.); ^{1}H nmr (90 MHz, dmso-d_6): δ 3.84 (s, 3H, OCH_3), 7.12 (d, J=9 Hz, 2H, ArH), 8.21 (d, J=9 Hz, 2H, ArH), 8.28 (s, 1H, H-2), 9.52 (s, 1H, H-7); ms: m/e 254 (M^+, 100), 239 (7), 227 (M^+-27,6), 211 (6), 134 (M^+-120,17), 133 (37).

Anal. Calcd for $C_{13}H_{10}N_4O_2$ (254.24): C, 61.41; H, 3.96; Found : C, 61.20; H, 3.91.

*7-Aryl-4(3H)-pteridinone (**2a-c**)*

To a hot solution of 4,5-diamino-6(1*H*)-pyrimidinone (2.52 g, 20 mmol, mp. 238-40°) [35] in 60 ml of water being brought to pH=7.5 with solid sodium bicarbonate, was added a solution of 25 mmol of the appropriate arylglyoxal **4a-c** dissolved in 100 ml of ethanol:water (1:1) adjusted to pH=7.5 with aqueous sodium hydroxide. After stirring under gentle reflux for three hours with regular control to keep the pH=7.5, the hot solution is cooled. The (feathery) precipitate was filtered and successively washed with water, ethanol and ether. After drying over phosphorus pentoxide at 100° the yield of the crude product was about 80-90%. The product was recrystallized twice from dimethylsulfoxide [13] at 100°.

*7-Phenyl-4(3H)-pteridinone (**2a**)*

Yield 54%, colourless plates; mp > 345° (lit. [11] > mp 295° dec.). The mass and infrared spectrum of this product were identical with that obtained by oxidation of 7-phenylpteridine with m-chloroperbenzoic acid [11]. ^{1}H nmr (90 MHz, dmso-d_6): δ 7.44-7.70 (m, 3H, ArH), 8.15-8.45 (m, 2H, ArH), 8.33 (s, 1H, H-2), 9.33 (s, 1H, H-6); ms : m/e 224 (M^+, 100), 121 (60), 104 (M^+-120, 15).

Anal. Calcd for $C_{12}H_8N_4O$ (224.22): C, 64.28; H, 3.60. Found: C, 64.26, H; 3.37.

*7-(p-Methylphenyl)-4(3H)-pteridinone (**2b**)*

Yield 38%, white feathery short needles; mp > 335°; ^{1}H nmr (90 MHz, dmso-d_6): δ 2.47 (s, 3H, CH_3), 7.43 (d, J=7.5 Hz, 2H, ArH), 8.17 (d, J=7.5 Hz, 2H, ArH) 8.30 (s, 1H,H-2), 9.31 (s, 1H, H-6); ms : m/e 238 (M^+, 100),121 (44), 118 (M^+-120, 24).

Anal. Calcd for $C_{13}H_{10}N_4O$ (238.24) : C, 65.53; H, 4.23. Found: C, 65.28; H, 4.33.

*7-(p-Methoxyphenyl)-4(3H)-pteridinone (**2c**)*

Yield 42%, bright yellow short needles; mp 323-325° dec (lit. [11] > mp 320°; ^{1}H nmr (90 MHz, dmso-d_6): δ 3.93 (s, 3H, OCH_3), 7.15 (d, J=9 Hz, 2H, ArH), 8.28 (d, J=9 Hz, 2H, ArH), 8.33 (s, 1H, H-2), 9.37 (s, 1H, H-6); ms : m/e 254 (M^+, 100), 134 (M^+-120, 23), 121 (20).

Anal. Calcd for $C_{13}H_{10}N_4O_2$ (254.24): C, 61.40; H, 3.96. Found: C, 61.21; H, 4.25.

*3-Methylthiomethyl-6-phenyl-4-(3H)-pteridinone (**12**)*

A mixture of **10a** (0.25 g, 1.1 mmol), 5 ml of triethyl orthoformate and 5 ml of dimethylsulfoxide was heated under stirring at 110° for 100 hours. To the cooled solution 10 ml of water were added. The precipitate was filtered, washed with water, methanol and ether, and dried to give 0.2 g (64%) of a brown powder. Recrystallization from 1-propanol using charcoal yielded white-greenish fine needles, mp 197-8°; ^{1}H nmr (90 MHz, dmso-d_6): δ 2.27 (s, 3H, CH_3), 5.23 (s, 2H, CH_2), 7.50-7.70 (m, 3H, ArH), 8.15-8.35 (m, 2H, ArH), 8.77 (s, 1H, H-2), 9.66 (s, 1H, H-7); ms: m/e 284 (M^+, 61), 269 (M^+-15, 98), 238 (M^+-CH_2S, 72), 225 (100); exact mass measurement for $C_{14}H_{12}N_4OS$ (M^+) 284.0731 (Theoretical 284.0732).

Anal. Calcd for $C_{14}H_{12}N_4OS$ (284.33): C, 59.13; H, 4.25. Found: C, 59.11; H, 4.08.

*7-Aryl-2,4(1H,3H)-pteridinedione (**14a-e**)*

These compounds were prepared according to the method of Pfleiderer and Hutzenlaub [36] given for the preparation of the 7-phenyl derivative. To a solution of (3.57 g, 20 mmol) of 5,6-diaminouracil hydrochloric acid salt [48] in 60 ml of water, a solution of 25 mmol of freshly distilled arylglyoxal **4a-e** in 60 ml of ethanol was added. After stirring for 30 minutes at room temperature a yellow precipitate was obtained, which was collected on a Buchner, and washed well with water. The precipitate was dissolved in 500 ml of water (for

14c 1500 ml was used) with solid potassium hydroxide (pH=11). The solution was refluxed for about seven minutes. After treatment with charcoal and refluxing another five minutes the solution was filtered hot and still hot acidified with glacial acetic acid. After cooling the precipitate was collected by filtration, washed well with water and dried at 120° with phosphorus pentoxide. The products were chromatographically and analytically pure (tlc, solvent B and C).

*7-Phenyl-2,4(1H,3H)-pteridinedione (**14a**)*

Yield 70%; bright white chunky crystals, mp > 350° (lit. [16b,36] mp > 350°); ^{1}H nmr (90 MHz, dmso-d_6): δ 7.50-7.73 (m, 3H, ArH), 8.10 - 8.36 (m, 2H, ArH), 9.17 (s, 1H, H-6); ms : m/e 240 (M^+).

Anal. Calcd for $C_{12}H_8N_4O_2$ (240.22): C, 60.00; H, 3.36. Found: C, 59.79; H, 3.17.

*7-(p-Methylphenyl)-2,4(1H,3H)-pteridinedione (**14b**)*

Yield 63%; off-white chunky crystals, mp > 350°; ^{1}H nmr (90 MHz, dmso-d_6): δ 2.48 (s, 3H, CH_3), 7.43 (d, J=7.5 Hz, 2H, ArH), 8.15 (d, J=7.5 Hz, 2H, ArH), 9.12 (s, 1H, H-6), 11.65 (br s, 1H, NH), 11.92 (br s, 1H, NH); ms : m/e 254 (M^+).

Anal. Calcd for $C_{13}H_{10}N_4O_2$ (254.24): C, 61.40; H, 3.96. Found C, 61.18; H, 3.97.

*7-(p-Methoxyphenyl)-2,4(1H,3H)-pteridinedione (**14c**)*

Yield 55%; yellow chunky crystals, mp > 340° dec; ^{1}H nmr (90 MHz, dmso-d_6): δ 3.87 (s, 3H,OCH_3), 7.12 (d, J=9 Hz, 2H, ArH), 8.19 (d, J=9 Hz, 2H, ArH), 9.07 (s, 1H, H-6), 11.58 (br s, 1H, NH), 11.83 (br s, 1H, NH); ms : m/e 270 (M^+).

Anal. Calcd for $C_{13}H_{10}N_4O_3$ (270.24): C, 57.77; H, 3.73. Found : C, 57.65; H, 3.74.

Growth of Cells

Arthrobacter M-4 cells were grown as described elsewhere [9]. The cells were washed with phosphate buffer pH 7.2 (I=0.01) containing 0.1 mM EDTA. The four liter portion was resuspended in 200 ml of this buffer and divided into portions of 2.5 ml and stored frozen (-25°) until use for the immobilization or for kinetic assays.

Preparative scale conversion with xanthine oxidase (AXO).

Both the immobilization and oxidation procedure were carried out avoiding normal levels of room- and daylight.

Immobilization. Prior to immobilization the amount of portions needed for the reaction were lyophilized. The immobilization procedure was the same as that reported for *Arthrobacter* X-4 [7]. The lyophilized powder was suspended in 10% gelatin at 50° (dry weight ratio of cells and gelatin is one). This solution was immediately frozen in liquid nitrogen and lyophilized. The freeze-dried materials were carefully ground in a mortar and then added to a vigourously stirred 1% glutaraldehyde solution (1 ml of 25% glutaraldehyde was diluted with 11.5 ml of water and 12.5 ml of acetone) and stirring was continued for 30 minutes at room temperature. The off-white powder darkened during this time. Using 0.5% of glutaraldehyde solution instead of a 1% glutaraldehyde solution did not lead to a stable immobilized water insoluble matrix. The immobilized enzyme preparation was immediately packed in a column and washed with potassium phosphate buffer pH 8.0 (I=0.01, 0.1 mM EDTA) at 4° overnight.

Oxidation. For conversion of about 50 mg of **1a-c** 24 units were used and for 30-35 mg of **2a-c** 14 units. The product was dissolved with the minimal amount of 4N sodium hydroxide and diluted with potassium phosphate buffer pH 8.0 (I=0.01, 0.1 mM EDTA). A solution of 1000 ml (0.2 mM for **1a-c**) or 1200 ml (0.1 mM for **2a-c**) was slowly passed through the column at 20° and with a velocity of 0.5 ml/min. The conversion of substrate was followed by dilution of an aliquot in 0.1 N sodium hydroxide and measuring the UV-spectrum between 200-400 nm. Conversion of **1a-c** was complete within 120 hours of reaction while the oxidation of **2a** was complete after 90 hours. When the reaction was completed the column was run dry. The collected effluent was evaporated to a volume of about 100-150 ml and acidified with hydrochloric acid. The precipitate was collected by filtration and washed with distilled water. If necessary reprecipitation from an alkaline solution with acetic acid followed by filtration gave analytical pure products. The yields of the crude products and the analytical data of the purified products are given in Table 2.4.

Kinetic assays

The assay for *Arthrobacter* xanthine oxidase was performed as follows. Aliquots of 2.5 ml of frozen bacterial suspension were disrupted by ultrasonification with a Branson Sonifier B-12 (Branson Sonic Power Company, Danbury,

Connecticut) during six times 30 seconds at 36 W in an ice bath, taking care of keeping the temperature below 4°. This solution was centrifuged during 30 minutes at 5000 g. This almost clear solution was assayed for protein content in duplicate by the method of de Bard and Moss [49] using their modification of the method of Lowry, as well as for enzyme activity in triplicate using 100 μM xanthine and 100 μM 1-methylxantine at pH=7.2 [50]. For this bacterial enzyme, one unit of enzyme activity is the amount of enzyme which oxidizes 1 μ mol of 1-methylxanthine per min at 25°C. The assay conditions were: 100 μM substrate in 50 mM potassium phosphate buffer, pH=7.2, including 0.1 mM EDTA with the reaction monitored at 292 nm (log $\Delta\varepsilon$=4.09) for the 1-methylxanthine and at 269 nm (log $\Delta\varepsilon$=3.89) for xanthine, using oxygen as the final electron acceptor. The rate was determined from the initial slope of the absorbance *versus* time, representing the rate of the disappearance of the substrate.

Kinetic parameters were estimated by the method of Naqui and Chance [51]. This method was used because at the applied protein concentration the molar differential absorption coefficient was dependent on the used substrate concentration. Only when the crude cell-free extract was diluted the law of Lambert-Beer was obeyed again. The assay mixture contained oxygen as the final electron acceptor. As buffer, Tris-HCl, pH=8.0 with an ionic strength I=0.05, including 0.1 mM EDTA was used and the substrate to be oxidized at the appropriate concentrations in a final volume of 2 ml. Each assay (at least performed in duplicate) was initiated by addition of 0.05 ml of cell-free extract (approx. 3.8 mg/ml) in potassium phosphate (I=0.01, pH=7.2). The temperature of the assay mixture was maintained at 25°. The oxidation of the substrates was determined at a suitable wavelength using a DMS 100 spectrophotometer coupled with a DS 15 data station. The rate was determined from the time to exhaust half of the initial substrate concentration as a function of the initial substrate concentration [51]. The appropriate wavelengths (λ in nm), the corresponding mean molar differential absorption coefficients (log $\Delta\varepsilon$) and substrate concentration range are: **1a-13a:** 336 (3.53) from 5 to 50 μM **2a-14a:** 365 (3.67) from 5 to 50 μM and **15-16:** 375 (3.23) from 30 to 330 μM. Kinetic data were calculated from Hanes-Woolf plots [51,52].

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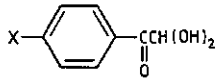

I

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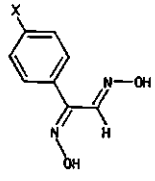

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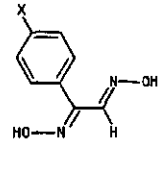

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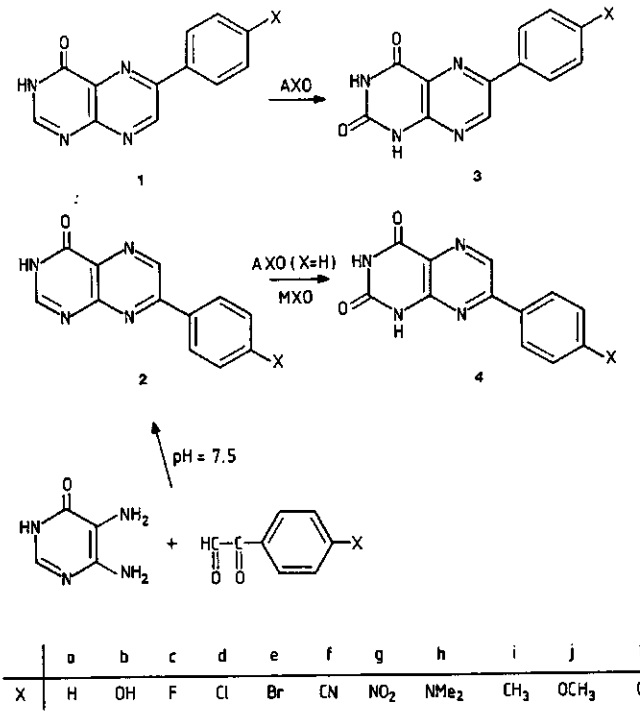
3 THE BEHAVIOUR OF 6-ARYL-4(3H)-PTERIDINONES AND 7-ARYL-4(3H)-PTERIDINONES TOWARDS XANTHINE OXIDASE FROM ARTHROBACTER M-4

3.1 INTRODUCTION

Recently the oxidation of 6-aryl-4(3*H*)-pteridinones **1** into the corresponding 6-aryl-2,4(1*H*,3*H*)-pteridinediones (6-aryllumazines) **3** by xanthine oxidase from *Arthrobacter* M-4 (AXO) has been described [1]. Using the same enzyme system, 7-phenyl-4(3*H*)-pteridinone (**2a**) is converted into 7-phenyllumazine (**4a**) (Scheme 3.1); the initial maximal velocity (Vm) of oxidation is about half of that of 6-aryl-4(3*H*)-pteridinone **1a**. In the 6- and 7-phenyl derivatives as well as in the parent system 4(3*H*)-pteridinone the AXO-mediated oxidation exclusively takes place at C-2 in contrast to bovine milk xanthine oxidase (MXO) which oxidizes 4(3*H*)-pteridinone first at C-7 and then at C-2 [2].



	a	b	c	d	e	f	g	h	i	j	k
X	H	OH	F	Cl	Br	CN	NO_2	NMe_2	CH_3	OCH_3	CF_3

Scheme 3.1

Although the oxidation rate for 4(3*H*)-pteridinone was higher than for the phenyl derivatives the affinity of the 6- and 7-phenyl-4(3*H*)-pteridinone **1a** and **2a** for AXO is enhanced as expressed by their lower Km-value [1]. The presence of hydrophobic group(s) in the active site of the enzyme favours the interaction of substrates possessing the hydrophobic phenyl group [3]. Due to this interaction 6-aryl-4(3*H*)-pteridinones **1** act as better inhibitors for MXO than allopurinol [4].

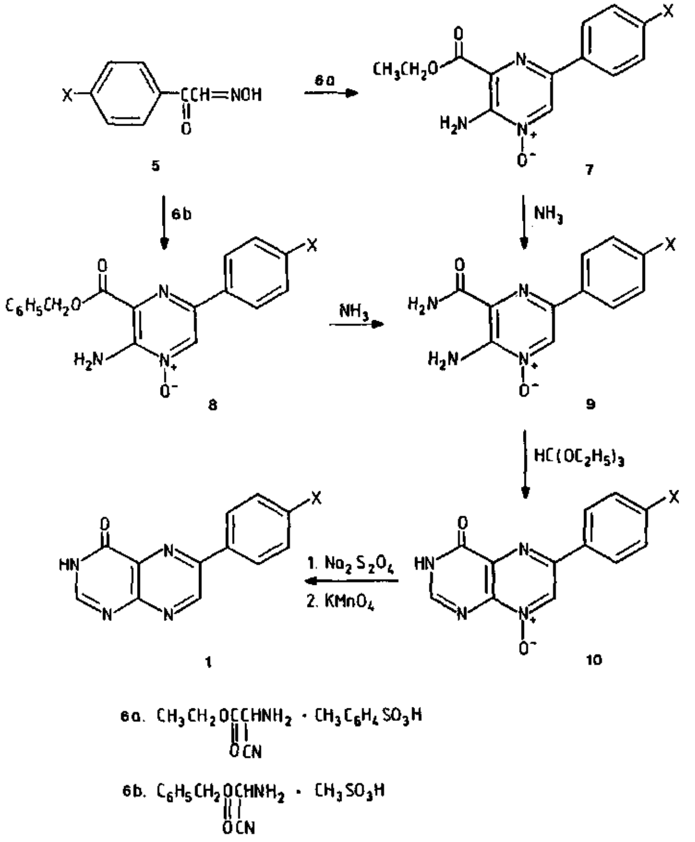
Earlier investigations have revealed that 7-aryl-4(3*H*)-pteridinones are easily oxidized by MXO into the corresponding lumazines [5]. However, these compounds show to be poor substrates in the AXO-mediated reactions [1]. A study of the electronic effect of the substituent at the para position of the 7-aryl-4(3*H*)-pteridinones on the oxidation rate with milk enzyme demonstrate that electron-donating substituents enhance the velocity of oxidation in contrast with electron-withdrawing groups, which lower the rate. From the slope of the Hammett plot (σ *versus* log Vm) a σ-value of about - 0.5 was calculated for both the free and immobilized bovine milk xanthine oxidase, indicating a definitive but rather small influence of the para substituent [5].

A study was initiated in order to investigate if in the AXO-mediated oxidation of 6-aryl-4(3*H*)-pteridinones **1** an electronic effect of the para substituent in the aryl group on the oxidation rate could be found. The present report deals with attempts to synthesize a series of 6-(*p*X-phenyl)-4(3*H*)-pteridinones and the effect of the para substituent on the maximal velocity (Vm) after incubation with AXO. In this study we also included the inhibitory effect of 7-aryl-4(3*H*)-pteridinones **2a-e, 2g, 2i, 2j** and **2k** on the oxidation of 1-methylxanthine into 1-methyluric acid by the bacterial xanthine oxidase.

3.2 SYNTHESIS OF 6- AND 7-ARYL-4(3*H*)-PTERIDINONES

*3.2.1 7-Aryl-4(3*H*)-pteridinones*

The 7-aryl-4(3*H*)-pteridinones **2a-e, 2g, 2i, 2j** and **2k** were prepared in high yield (80 - 90%) by a Gabriel-Isay condensation [6]. This procedure, involving 4,5-diamino-6(1*H*)-pyrimidinone and arylglyoxals, could potentially yield some of the 6-aryl isomer. However, when using proper conditions, *i.e.* performing the condensation at pH=7.5 and applying a selective recrystallization solvent (DMSO), the 7-aryl-4(3*H*)-pteridinone are obtained almost without contamination of the corresponding 6-aryl isomer [1,4,5].



	a	b	c	d	e	f	g	h	i	j
X	H	OH	F	Cl	Br	CN	NO_2	NMe_2	CH_3	OCH_3

Scheme 3.2

3.2.2 6-Aryl-4(3H)-pteridinones

The synthesis of the compounds **1a**, **1i**, **1j** using the method of Taylor and coworkers [7,8] (Scheme 3.2) has recently been published [1]. The key intermediate in the synthesis of 6-aryl-4(3*H*)-pteridinones is 2-amino-3-ethoxycarbonyl-5-arylpyrazine-1-oxide (**7**), which was obtained through condensation of ethyl 2-amino-2-cyanoacetate *p*-toluenesulfonic acid salt **6a** [1] and arylglyoxal-2-oximes **5** in absolute methanol at 35°. The reaction time and yields obtained for the products **7** when using about one equivalent or a sligth excess of α-aminonitril **6a**, are listed in Table 3.1. From this Table it is clear that the more electron-withdrawing the substituent X at the para position is, the

Table 3.1 Reaction conditions and yields for the preparation of 2-amino-3-ethoxycarbonyl-5-arylpyrazine-1-oxides 7 and 2-amino-3-benzyl-oxycarbonyl-5-arylpyrazine-1-oxides 8.

Starting Compound	Product	Ratio [a]	Time	Yield	Product	Ratio [b]	Time	Yield
5a	**7a** [c]	1.0	50 h	77%	**8a** [c]	1.0	120 h	90%
5b	**7b**	1.0	170 h	67%	**8b**	1.0	170 h	72%
5c	**7c**	1.0	240 h	51%	**8c**	1.0	215 h	73%
5d	**7d**	1.0	240 h	62%	**8d**	1.0	240 h	74%
5e	**7e**	1.25	360 h	79%	**8e**	1.0	400 h	70%
5f	**7f**	1.0	960 h	59% [d]	**8f**	1.0	240 h	27%
						1.0	500 h	40%
5g	**7g**	1.33	500 h	32%	**8g**	1.35	525 h	56%
		1.0	1370 h	52% [d]				

[a] Using this ratio of **6a** and corresponding **5**, the reaction was performed in absolute methanol at 35 °.

[b] The reactions were performed at room temperature using the indicated ratio of **6b** and appropiate **5**.

[c] Compounds **7a** and **8a** have already been described [7,8].

[d] The isolated product contained as large amount of p-toluenesulfonic acid.

longer reaction time is needed in order to obtain **7**. When X = F, Cl or Br about ten days are required to obtain moderate yields (50 - 70%) of **7c**, **7d** and **7e**, respectively, but in the case of α-hydroxyimino-*p*-cyanoacetophenone **5f** and α-hydroxyimino-*p*-nitroacetophenone **5g** the reaction time had to be prolonged till about one month. The strong mesomeric interaction between the nitro or cyano group and the keto group retards the initial reaction between the amino group of the α-aminonitril **6a** and the ketogroup of the arylglyoxal-2-oximes **5**. The subsequent reaction of the oxime group of **5** with the carbon of the cyano group of **6a** terminates the cyclization of the resulting 5-arylpyrazine-1-oxides **7** [9].

Since during the reaction between **5f**, **5g** and **6a** *p*-toluenesulfonic acid deposited together with the desired compounds **7f** and **7g**, condensation of benzyl 2-amino-2-cyanoacetate methanesulfonic acid salt **6b** and **5** was generally preferred. The resulting 2-amino-3-benzyloxycarbonyl-5-arylpyrazine-1-oxides **8** are

more suitable as key intermediates since their work-up and isolation procedure does not lead to undesirable contamination of side products. Moreover, the yields in which the benzylesters **8** are obtained, are usually higher than those obtained for the ethylesters **7** (Table 3.1) [1].

However, when a dimethylamino group is present in the para position, *i.e.* **5h**, this compound is not able to undergo ring closure with either **6a** or **6b** to the ester **7h** or **8h** not even when using other α-aminonitrils such as 2-amino-cyanoacetamide [10] and aminomalononitrile [11] and/or other solvents (pyridine, chloroform, 2-propanol, acetic acid).

Aminolysis in liquid ammonia [1,12] of both the ethylesters **7b-g** and benzylesters **8b-g** afforded 2-amino-3-carbamoyl-5-arylpyrazine-1-oxides **9b-g**, virtually in quantitive yield. Subsequent ring closure of the amides **9b, 9d** into the 6-aryl-4(3*H*)-pteridinones-8-oxides **10b, 10d** could be achieved in moderate yield using triethyl orthoformate in dimethylformamide.

In the reaction of **9f** into **10f** a dark brown mixture was obtained from which no product could be isolated. The cyclization of **9g** yielded an orange-yellow precipitate in about 25% yield. The low-energy mass spectrum of this compound showed, besides the parent peak m/e 313, a peak m/e 269, suggesting the formation of 6-(*p*-nitrophenyl)-4(3*H*)-pteridinone and a peak m/e 297, suggesting the formation of 3-ethyl-6(*p*-nitrophenyl)-4(3*H*)-pteridinone. Exact mass measurement for peak m/e 313 gave an experimental value of 313.0822 (theoretical 313.0811 for $C_{14}H_{11}N_5O_4$) and thus suggesting 3-ethyl-6(*p*-nitrophenyl)-4(3*H*)-pteridinone-8-oxide as the main product, and two side products. The 300 MHz nmr spectrum revealed the signals: δ 1.40 (t,3H,CH_3), 4.14 (q,2H,CH_2), 8.52 -8.56 (m,4H,ArH), 8.75 (s,1H,H-2), 9.62 (s,1H,H-7) indicating that ring closure of **9g** almost exclusively gives 3-ethyl-6-(*p*-nitrophenyl)-4(3*H*)-pteridinone-8-oxide [13] and that the desired compound **10g** was not obtained in this way.

The pteridinone-8-oxides **10b, 10d** could be deoxygenated into the desired 6-aryl-4(3*H*)-pteridinones **1b, 1d** by treatment with sodium dithionite. This procedure not only leads to deoxygenation but also to hydrogenation of the pyrazine ring [8], mild oxidation with potassium permanganate solution is necessary to convert the partly reduced 6-aryl-4(3*H*)-pteridinones into **1**.

3.3 ENZYMATIC OXIDATION AND INHIBITION

3.3.1 7-Aryl-4(3H)-pteridinones

The cell-free extract of *Arthrobacter* M-4 was prepared in the usual manner

as described earlier [1]. The crude enzyme extract was used to determine the susceptibility of the aryl-4(3*H*)-pteridinones towards oxidation by AXO. In a recent paper [1] we have already reported that 7-(*p*-methylphenyl)- and 7-(*p*-methoxyphenyl)-4(3*H*)-pteridinones **2i** and **2j** respectively are oxidized by AXO at pH=7.2 and pH=8.0. However their rate is very low in comparison with **2a**. When 50 and 100 μM solutions of **2b, 2c, 2d, 2f** and **2g** were incubated with AXO at both pH-values and the reaction was monitored by UV-spectroscopy, no oxidation could be observed for the compounds **2b, 2d, 2f** and **2g**. Only compound **2c** was oxidized at C-2 and the rate of the oxidation was about the same as for **2a**. The similarity of the UV-spectrum of **2a** with that of **2c** and those of their respective oxidation products indicated that the final product was 7-(*p*-fluorophenyl)-2,4(1*H*,3*H*)-pteridinedione.

In AXO mediated reactions the 7-aryl-4(3*H*)-pteridinones **2** are not or only very slowly oxidized. An indication of the binding properties of these substrates towards the bacterial enzyme on an equal base in order to make comparison possible on an equal base could be obtained by measuring the I_{50}-value of these compounds at pH=7.25 using 100 μM of 1-methylxanthine as substrate. The results of these experiments are summarized in Table 3.2.

Table 3.2 *I_{50}-values* [a] *for the inhibition of 100 μM of 1-methylxanthine by 7-aryl-4(3H)-pteridinones* **2** *at pH=7.25 using the cell-free extract of AXO* [b] .

Compound	I_{50} [a]
2a	9.2 ± 0.7
2b	4.7 ± 0.3
2c	10.5 ± 0.9
2d	12.3 ± 1.0
2g	32.6 ± 3.9
2i	6.7 ± 0.3
2j	5.8 ± 0.6 [c]
2k	22.9 ± 2.1

[a] In μmol/l.

[b] Under standard conditions the activity of AXO was 0.29 ± 0.03 μ mol/min.mg^{-1} protein.

[c] This compound was assayed thrice.

When the I_{50}-values from Table 3.2 are plotted as function of the σ-value of the para substituent a nearly straight line is obtained (Figure 3.1). The slope of this line is about + 0.73. This slope is quite significant and clearly illustrates that the strength of inhibition on the oxidation rate of 1-methylxanthine into 1-methyluric acid is dependent on the nature of the para substituent of the 7-aryl-4(3*H*)-pteridinones.

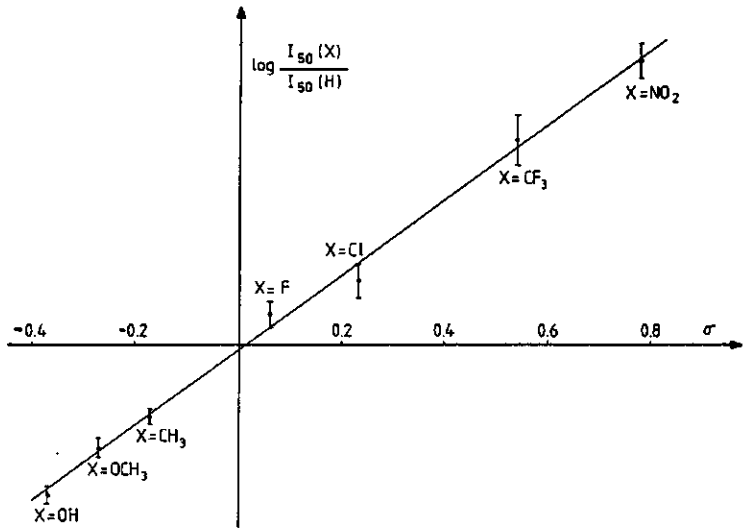
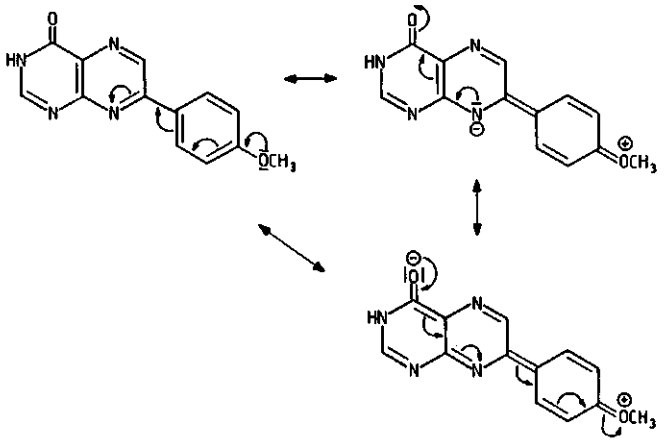


Figure 3.1 *Plot of the logarithm of the ratio of the I_{50}-value of the 7-(p-phenyl)-4(3H)-pteridinones and 7-phenyl-4(3H)-pteridinones* versus *the substituent constant σ of X of the inhibition of 1-methylxanthine into 1-methyluric acid.*

With electron-donating groups i.e. **2b** and **2j** the inhibition is strong. The highest I_{50}-values are observed for **2f**, **2g** and **2k** possessing electron-withdrawing groups which indicates that the strength of inhibition is much weaker. As can be seen from Scheme 3.3 an electron-donating substituent delocalizes its electron pair over both rings [14] enhancing the electron density on oxygen and N-8, apparently favouring binding to the enzyme. From the resonance structures in Scheme 3.3 it can be seen that N-8, which probably is involved in binding at the Mo cofactor [5], has an increased electron density

for **2b**, **2j**.



Scheme 3.3

Bunting has recently demonstrated that in bovine milk xanthine oxidase, interaction of the C=O moiety of the substrate with a (proton donating) species in the active site of the enzyme is an important factor in the determination of the orientation of substrate molecules in the active site of the enzyme [15]. In the bacterial enzyme obviously the C=O group of the substrate is also involved in binding with a (proton donating) species in the active site since an increase of electron density at C=O gives a better inhibition. From the results obtained in this study it seems that (i) both the C=O group at C-4 and N-8 in 7-aryl-4(3*H*)-pteridinones are important binding sites and that (ii) slowly oxidizable substrates are good inhibitors: a substituent larger than hydrogen or fluor at the para position in the phenyl ring transforms good substrates into compounds with inhibitory capacities.

3.3.2 6-Aryl-4(3H)-pteridinones

When 6-(*p*-hydroxyphenyl)-4(3*H*)-pteridinone **1b** and 6-(*p*-chlorophenyl)-4(3*H*)-pteridinone **1d** were subjected to incubation with AXO at pH = 7.2 and 8.0 they were quickly converted into the corresponding 6-aryl-2,4-(1*H*,3*H*)-pteridinediones (6-aryllumazines) as observed by measuring the reaction with UV-spectroscopy at regular intervals. An overlay of these spectra exhibited several isosbestic points, indicating a clean reaction which leads to the formation of a stable product. The final spectrum was identical to that of an authentic

sample of 6-aryl-2,4(1*H*,3*H*)-pteridinediones **3** prepared independently.

Arthrobacter M-4 cells immobilized in gelatine crosslinked with glutaraldehyde [1,16] were used to perform small preparative oxidations of **1b** and **1d**. After 120 hours of incubation with immobilized cells the corresponding 6-aryllumazines **3b** and **3d** were obtained in a yield of 94% and 89%, respectively.

Table 3.3 Kinetic parameters for the oxidation of some 6-aryl-4(3H)-pteridinones 1 by the crude cell-free extract of AXO at pH=8.0 [a].

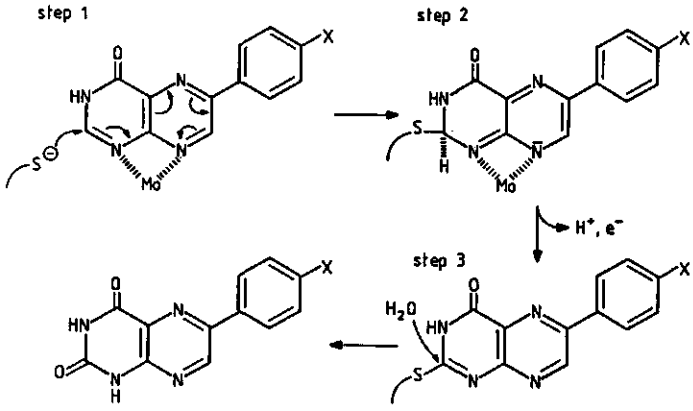
Compound	Km [b]	Vm [c]	Vm/Km [d]
1a	11.9 ± 1.2	0.112 ± 0.011	9.4
1b	7.0 ± 0.5	0.116 ± 0.006	16.6
1d	11.5 ± 0.8	0.108 ± 0.009	9.4
1i	13.8 ± 1.4	0.105 ± 0.012	7.6
1j	9.0 ± 0.9	0.115 ± 0.010	12.8

[a] The oxidation rate for 1-methylxanthine with the bacterial xanthine oxidase preparations used here was 0.29 ± 0.03 μmol/min.mg^{-1} protein.
[b] In μmol/l.
[c] In μmol/min.mg^{-1} protein.
[d] In ml/min.mg^{-1} protein.

In order to study the influence of aryl substituents on the oxidation rate with AXO, the kinetic parameters Km and Vm were estimated for the compounds **1a**, **1b**, **1d**, **1i** and **1j** at pH=8.0 (Table 3.3). The influence of substituent X on the initial maximal oxidation rate at C-2 by this enzyme is remarkable small. This result is in contrast to the observations for the oxidation of 7-aryl-4(3*H*)-pteridinones by bovine milk xanthine oxidase, which clearly shows an activating effect of electron donating groups at the para position [5].

Based on the mechanism proposed by Robins [17] for the MXO-mediated oxidation of hypoxanthine into xanthine an analogous *Type II Binding* [17] for the 6-aryl-4(3*H*)-pteridinones is put forward (Figure 3.3). In the oxidation of **1** into the corresponding 6-aryllumazines the following series of events [5,17,18] takes place : (step 1) the substrate molecule is attached to the active site of the enzyme in such a way that the nucleophilic sulfide ion can attack the reaction center, *i.e.* C-2 of the pteridine ring, (step 2) from the formed covalent complex a hydride equivalent is transferred to the molybdenum

cofactor, (step 3) the covalent bond between sulfur and the substrate is attacked by a water molecule which results in the liberation of the oxidized substrate and the reduced enzyme. Release of the electrons from Mo(IV) to the final electron acceptor reoxidizes the Mo cofactor to the Mo(VI) state.



Scheme 3.4

From the structures in Figure 3.2 can be seen that the para substituent with the aryl group at C-6 has a direct mesomeric effect on the C-2 reaction center of the 6-aryl-4(3*H*)-pteridinones: electron-donating substituents will exhibit a retarding effect while electron-withdrawing groups will give an accelerating effect on the rate of the nucleophilic addition of at C-2. It is also visualized that coordination of Mo to the ring nitrogens N-1 and N-8 will result in an enhanced electrophilicity at C-2 [19]. If the nucleophilic attack by either the sulfide at C-2 in the first step or water in the third step would be rate determining a lower rate is expected for increasing electron donation from the aryl moiety [5]. However no effect of variation of X is observed.

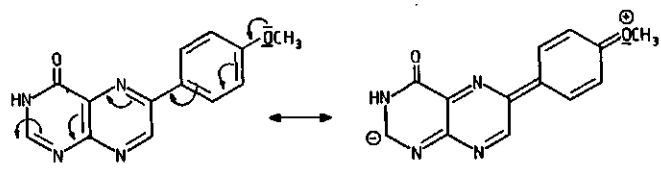
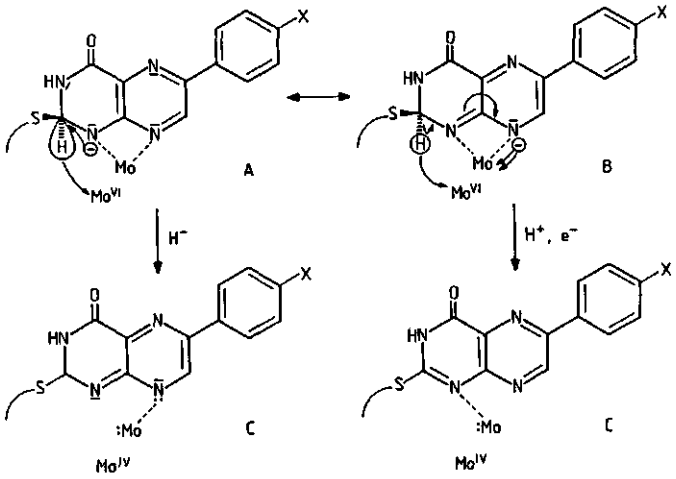


Figure 3.2 Resonance structures of 6-(p-methoxyphenyl)-4-(3H)-pteridinone.

This result can best be reconciled with the assumption that the second step is rate-limiting. In this step variation of substituents in the aryl moiety of the position 6 of the pteridine ring can be only of minor importance because no mesomeric interaction with the reaction center is possible. In step 2 of Scheme 3.4 a C-H bond is broken. Generally C-H bond cleavage is a relatively slow process and there are numerous examples in (bio)organic chemistry in which C-H bond breaking is the rate-limiting step [20].



Scheme 3.5

In principle electron transfer from the substrate towards Mo may proceed (i) as a concerted proton/two electron transfer or (ii) as a hydride transfer [21]. In mechanism (i) a proton is removed from carbon and the resulting negative charge on carbon must be transferred for a large part to a more electronegative atom in the molecule. In mechanism (ii) a hydride is removed from C-2 and the incipient positive charge on carbon has to be compensated by the transfer of an electron pair to this carbon atom. Both processes bring about strong polarization in the molecule which results in a relative high free energy of activation. Althought there is no direct experimental evidence in favor of one of both mechanisms we prefer the simultaneous proton/electron pair transfer since in this mechanism the function of Mo is much more unambiguous then in the hydride tranfer mechanism. From the structure of the covalent adduct formed after the addition of the thiolate group (Structure B in Scheme 3.5) it can be seen that in the first step Mo is liganded by its coordination

with the free electron pairs of the nitrogens in the pteridine ring. This acts catalytically on nucleophilic attack of the thiolate group since Mo has enabled approach of the nucleophile. The negative charge in the ring is delocalized over N-1 and N-8. In the same role Mo can accept the electron pair from one of the nitrogen atoms almost simultaneously with proton abstraction from C-2. The alternative mechanism (ii) however requires a very ambivalent role of Mo. Stabilization of structure A (Scheme 3.5) requires electron pair acceptation from nitrogen. However, the transfer from a hydride ion requires release of the free electron pair to the nitrogen and uptake of the electron pair of the hydride. It is not easy to see what would be the driving force for Mo to take part in such a process. Furthermore it is not clear in this mechanism which group in the active site would be able to accept a hydride. It has been established that the hydrogen atom becomes ultimately attached to a liganting oxygen of Mo [22].

For both mechanisms, however, the aryl moiety in 6-aryl-4(3*H*)-pteridinones is unable to exert large electronic effects. Direct mesomeric electron donation to C-2 is not possible due to its sp^3 hybridization. Also the neighbouring atoms at C-2, *i.e.* N-1 and N-3 are not subjected to mesomeric electron donation by the aryl group. The inductive effect will be rather small since the distance of substituents and the reaction center is rather large.

Differences in substrate specificity between bovine milk xanthine oxidase and bacterial xanthine oxidase are probably mainly related to differences in the hydrophobic site of the both enzymes [23]. In comparison with MXO, AXO has a relatively less hydrophobic pocket in the active site. As seen from Table 3.3 the Km-values for AXO for various substrates are different: **1b** and **1j** bind more easily than the other ones. These are the substrates with the most polar substituents, which are also capable of hydrogen bond acceptation. These properties might well be essential for strong binding to the active site as is also exemplified by the strong inhibitory capacity of 5,6-diaminouracil [24].

Recently a QSAR has been developed for 6-aryl-4(3*H*)-pteridinones which indicates that 6-aryl compounds with rod shaped alkyl substitutents at the para position are better inhibitors for MXO then spherical shaped substituents [4]. It is reasonable to expect that both AXO and MXO oxidize aryl-4(3*H*)-pteridinones by quite a similar mechanism since the enzymatic nucleophile in these enzymes has been found to act on C-2 and could produce the corresponding lumazines. However the rates of oxidation differ widely [1,4,5,23]. This is most likely attributed to differentiations in orientation of the substrates caused by differences in the hydrophobic pocket of the enzymes, which determine the

fate of the aryl-4(3*H*)-pteridinones.

Earlier work has revealed that 7-aryl-4(3*H*)-pteridinones **2** are oxidized at C-2 yielding the corresponding lumazines by treatment with bovine milk xanthine oxidase [5,25], while 6-aryl-4(3*H*)-pteridinones **1** are good inhibitors [4]. In this and other studies [1,16,23] it is shown that 6-aryl-4(3*H*)-pteridinones **1** are good substrates and in general the 7-aryl-4(3*H*)-pteridinones are good inhibitors for the bacterial xanthine oxidase from *Arthrobacter* M-4.

3.4 EXPERIMENTAL SECTION

Melting points were determined on a Kofler hot stage equipped with a microscope and a polarizer and they are uncorrected. ^{1}H nmr spectra were measured in deuterated dimethylsulfoxide solutions using a Varian EM 390 or a Bruker CXP 300 spectrometer equipped with a B-VT 1000 variable temperature controller with tetramethylsilane as internal standard. Mass spectra were recorded on a AEI 902 instrument equipped with a VG-ZAB console. Ultra violet spectra were determined using a Beckmann DU-7 or a Varian DMS 100 spectrophotometer equipped with a DS 15 data station. Column chromatography was carried out over Merck Silicagel 60 (70-230 mesh ASTM) and Silicagel GF from Merck was used for analytical thin layer chromatography.

Preparation of starting materials and reference compounds

p-Dimethylaminoacetophenone [26], *p*-hydroxyphenylglyoxal hydrate [27], *p*-fluorophenylglyoxal [28], *p*-chlorophenylglyoxal hydrate [29,30], *p*-bromophenylglyoxal [29,30], *p*-nitrophenylglyoxal [31], 2-amino-2-cyanoacetamide [11,32], 6-phenyl-4(3*H*)-pteridinone (**1a**) [1], 6-(*p*-methylphenyl)-4(3*H*)-pteridinone (**1i**) [1], 6-(*p*-methoxyphenyl)-4(3*H*)-pteridinone (**1j**) [1], 7-phenyl-4(3*H*)-pteridinone (**2a**) [1,5], 7-(*p*-methylphenyl)-4(3*H*)-pteridinone (**2i**) [1], 7-(*p*-methoxyphenyl)-4(3*H*)-pteridinone (**2j**) [1,5], 6-(*p*-chlorophenyl)-2,4(1*H*,3*H*)-pteridinedione (**3d**) [33], 5,6-diaminouracil hydrochloric acid salt [34], 7-(*p*-chlorophenyl)-2,4(1*H*,3*H*)-pteridinedione (**4d**) [33], ethyl 2-amino-2-cyanoacetate *p*-toluenesulfonic acid salt (**6a**) [1,35,36], benzyl 2-amino-2-cyanoacetate methanesulfonic acid salt (**6b**) [37], 2-amino-3-ethoxycarbonyl-5-phenylpyrazine-1-oxide (**7a**) [7,8], 2-amino-3-benzyloxycarbonyl-5-phenylpyrazine-1-oxide (**8a**) [1] were prepared according to known synthetic procedures and methods described in the literature. Aminomalononitrile *p*-toluenesulfonic acid salt was purchased from Janssen Chimica or Fluka.

General procedure for the preparation of 7-aryl-4(3H)-pteridinones **2**

To a hot solution of 4,5-diamino-6(1*H*)pyrimidinone (2.52 g, 20 mmol, mp. 238-40°) [38] in 50 ml of water brought to pH=7.5 with solid sodium bicarbonate was added a solution of 25 mmol of the appropiate arylglyoxal dissolved in 100 ml of ethanol:water (1:1) adjusted to pH=7.5 with aqueous sodium hydroxide. After stirring for three hours at gentle reflux the hot solution is cooled. The precipitate was filtered and successively washed with water, ethanol and ether. The yield of the crude product was about 80-90%. The product was recrystallized twice from dimethylsulfoxide at 100°.

*7-(p-Hydroxyphenyl)-4(3H)-pteridinone (**2b**)*

Yield 32%, chunky yellow crystals, mp. > 340° dec.

Anal. Calcd for $C_{12}H_8N_4O_2 * \frac{1}{2}H_2O$ (249.22): C, 57.83; H, 3.64; N, 22.48. Found : C, 57.66; H, 3.69; N, 22.17.

*7-(p-Fluorophenyl)-4(3H)-pteridinone (**2c**)*

Yield 42%, white needles, mp. > 335° dec.

Anal. Calcd for $C_{12}H_7FN_4O$ (242.21): C, 59.50; H, 2.91; N, 23.13. Found : C, 59.18; H, 2.99; N, 23.09.

*7-(p-Chlorophenyl)-4(3H)-pteridinone (**2d**)*

Yield 47%, white feathery needles, mp. > 345° dec.

Anal. Calcd for $C_{12}H_7ClN_4O$ (258.67): C, 55.72; H, 2.73; N, 21.66. Found : C, 55.64; H, 2.74; N, 22.08.

*7-(p-Nitrophenyl)-4(3H)-pteridinone (**2g**)*

Yield 35%, pale yellow powder, mp. > 350° dec.

Anal. Calcd for $C_{12}H_7N_5O_3$ (269.22): C, 53.53; H, 2.62; N, 26.02. Found : C, 53.65; H, 2.70; N, 26.21.

*7-(p-Trifluoromethylphenyl)-4(3H)-pteridinone (**2k**)*

Yield 51%, white short needles, mp. > 340° dec.

Anal. Calcd for $C_{13}H_7F_3N_4O$ (292.22): C, 53.43; H, 2.41; N, 19.17. Found : C, 53.16; H, 2.37; N, 19.03.

*6-(p-Hydroxyphenyl)-2,4(1H,3H)-pteridinedione (**3b**)*

The two-step method of Yoneda [39] was used to prepare this compound. A mixture of 5.4 g (30.3 mmol) 5,6-diaminouracil hydrochloric acid salt, 4.3 g

(35.2 mmol) *p*-hydroxybenzaldehyde and 60 ml of ethanol was refluxed during four hours and then cooled. The tan solid was filtered, washed with boiling water, ethanol and ether and dried in a yield of 57%. Recrystallization from dimethylformamide-water afforded almost colourless chunky crystals of 6-amino-5-(*p*-hydroxybenzylidene)amino-2,4(1*H*,3*H*)pyrimidinedione, mp. 320° dec.; ^{1}H nmr: δ 6.48 (br s,2H,NH_2), 6.75 (d,J=9 Hz,2H,ArH), 7.68 (d,J=9 Hz,2H,ArH), 9.55 (s,1H, CH=N), 9.70 (br s,1H,OH), 10.40 (br s,1H,NH) and 11.50 (br s,1H,NH).
Anal. Calcd for $C_{11}H_{10}N_4O_3*\frac{1}{2}H_2O$ (255.24): C, 51.76; H, 4.34; N, 21.95. Found : C, 52.01; H, 4.35; N, 22.25.

Cyclization of this compound (3.78 g, 15 mmol) in a mixture of 25 ml of triethylorthoformate and 25 ml of dimethylformamide gave after refluxing 16 hours in a yield of 84%. The yellow precipitate was recrystallized twice from dimethylformamide-water, mp. > 340° dec.; ^{1}H nmr: δ 6.92 (d,J=9 Hz,2H,ArH), 7.98 (d,J=9 Hz,2H,ArH), 9.15 (s,1H,H-6), 10.50 (br s,1H,OH), 10.90 (br s,1H,NH) and 11.60 (br s,1H,NH).
Anal. Calcd for $C_{12}H_8N_4O_3*2\ H_2O$ (292.25): C, 49.32; H, 4.14; N, 19.17. Found : C, 48.96; H, 3.95; N, 18.92.

*7-(p-Hydroxyphenyl)-2,4(1H,3H)-pteridinedione (**4b**)*

This compound was prepared according the method of Pfleiderer and Hutzenlaub [40]. To a solution of 5,6-diaminouracil hydrochloric acid salt (3.57 g, 20 mmol) in 60 ml of water, a solution of *p*-hydroxyphenylglyoxal hydrate [27] (30 mmol, 5.04 g) in 60 ml of ethanol was added. After stirring for 30 minutes at room temperature the yellow precipitate was collected on a Buchner. The precipitate was dissolved in 500 ml of water with solid potassium hydroxide (pH=11). The solution was refluxed for about five minutes, charcoal was added and heating was continued for an additional five minutes. The solution was filtered hot and still hot acidified with acetic acid. After cooling the yellow precipitate was collected by filtration, washed well with water and ethanol and dried at 120° with phosphorus pentoxide to give **4b** in a yield of 71%, mp. > 350°; ^{1}H nmr: δ 6.95 (d,J=9 Hz,2H,ArH), 8.12 (d,J=9 Hz,2H,ArH), 9.05 (s,1H,H-6), 10.25 (br s,1H,OH), 11.56 (br s,1H,NH) and 11.83 (br s,1H,NH).
Anal. Calcd for $C_{12}H_8N_4O_3$ (256.22): C, 56.25; H, 3.15; N, 21.87. Found : C, 56.06; H, 3.10; N, 21.95.

*General procedure for the preparation of arylglyoxal-2-oximes **5***

These compounds were prepared by the previous described two step method [1,37]. Para substituted acetophenones were oxidized using about 1.1 equivalent

of selenium dioxide in aqueous dioxane (95%) on a 0.2 - 0.33 mol scale using the presciptions from literature [41]. After refluxing 16 - 18 hours, the solution was filtered hot to remove the black precipitate. The yellow-orange solution was evaporated and the resulting oil was purified via column chromatography using chloroform as eluens. After evaporation of the appropiate fractions, a yellow oil was finally obtained. This oil could be used as such in the transoximation with acetonoxime. For characterization the oil was refluxed in twenty fold its weight in water during three hours. After the addition of charcoal the solution was filtered hot. The precipitate was isolated after standing overnight at 4° by filtration, washed well with ice-water and dried over phosphorus pentoxide, yielding the hydrate.

p-*Cyanophenylglyoxal hydrate*

Starting from 36.3 g (250 mmol) of *p*-cyanoacetophenone and 30.5 g (275 mmol) of selenium dioxide a yellow oil was obtained in a yield of about 90%. Conversion to the hydrate gave white chunky crystals, mp. 120-125°.
Anal. Calcd for $C_9H_5NO_2*\frac{1}{2}H_2O$ (168.15): C, 64.29; H, 3.60; N, 8.33. Found : C, 64.66; H, 3.81; N, 8.40.

p-*Dimethylaminophenylglyoxal hydrate*

After oxidation of *p*-dimethylaminoacetophenone (48.9 g, 0.3 mol) with selenium dioxide (40 g, 0.36 mol) the oil was immediately converted to its hydrate. This compound was obtained in a yield of 80% as a yellow powder, mp. 130-135°.
Anal. Calcd for $C_{10}H_{11}NO_2*H_2O$ (195.21): C, 61.52; H, 6.71; N, 7.18. Found : C, 61.25; H, 6.70; N, 7.17.

A mixture of either the solid hydrate or the oil (about 50 mmol) covered with 10 ml of methanol and 90 ml of water, was brought at pH=4. Acetonoxime (5.5 g, 75 mmol) was added and the solution was stirred at 50° during two hours. After cooling the precipitate was isolated by filtration. Recrystallization was achieved in methanol-water, unless otherwise indicated.

p-*Hydroxyphenylglyoxal-2-oxime (**5b**)*

The reaction mixture with acetonoxime was cooled and extracted thrice with 100 ml of chloroform. After drying with magnesium sulfate the combined chloroform layers were evaporated and the precipitate was recrystallized from water to give yellowish needles in 86% yield, mp. 160-3° (lit. [42], mp. 164-5°).

p-*Fluorophenylglyoxal-2-oxime (**5c**)*

Yield 92%, white needles, mp. 123-5° (lit. [43], mp. 128°).

p-*Chlorophenylglyoxal-2-oxime (**5d**)*

Yield 88%, after recrystallization from chloroform or methanol the white crystals melted at mp. 164-6° (lit. [44], mp. 158-60°).

p-*Bromophenylglyoxal-2-oxime (**5e**)*

Yield 93%, white needles, mp. 162-3° (lit. [44], mp.161°).

p-*Cyanophenylglyoxal-2-oxime (**5f**)*

Yield 89%, pale yellow needles, mp. 137-9°.

Anal. Calcd for $C_9H_6N_2O_2$ (174.15): C, 62.07; H, 3.47; N, 16.09. Found : C, 61.81; H, 3.69; N, 15.87.

p-*Nitrophenylglyoxal-2-oxime (**5g**)*

Yield 76%, yellowish plates, mp.140-1° (lit. [45], mp. 140°).

p-*Dimethylaminophenylglyoxal-2-oxime (**5h**)*

Yield 80%, yellow powder, mp.172-175°.

Anal. Calcd for $C_{10}H_{11}N_2O_2$ (192.21): C, 62.48; H, 6.29; N, 14.58. Found : C, 62.87; H, 6.31; N, 13.99.

*General procedure for the ring closure of arylglyoxal-2-oximes into the corresponding 2-amino-3-ethoxycarbonyl-5-arylpyrazine-1-oxides **7***

A mixture of the appropiate arylglyoxal-2-oxime (10 mmol) and 3.2 g, 4.0 g (in the case of **5e**) or 4.2 g (in the case of **5g**) of ethyl 2-amino-2-cyano-acetate *p*-toluenesulfonic acid salt [1] and 15 ml of absolute methanol was stirred at 35° during the time given in Table 3.1. After the solution was cooled to room temperature, the precipitate was filtered off and washed with cold ether (4°) and dried on the air in the yields also given in Table 3.1. Recrystallization was performed from 2-propanol unless otherwise indicated below.

*2-Amino-3-ethoxycarbonyl-5-(p-hydroxyphenyl)pyrazine-1-oxide (**7b**)*

Fine bright yellow needles, mp. 245-8°; ^1H nmr: δ 1.40 (t,J=7.5 Hz,3H,CH_3), 4.43 (q,J=7.5 Hz,2H,CH_2), 6.87 (d,J=9 Hz,2H,ArH), 7.55 (br s,2H,NH_2), 7.87 (d,J=9 Hz,2H,ArH), 9.03 (s,1H,H-6), 9.77 (br s,1H,OH).

Anal. Calcd for $C_{13}H_{13}N_3O_4$ (275.26): C, 56.72; H, 4.76; N, 15.27. Found : C, 56.66; H, 4.91; N, 15.37.

2-Amino-3-ethoxycarbonyl-5-(p-fluorophenyl)pyrazine-1-oxide (7c)

Fluffy bright yellow needles, mp. 171-3°; ^{1}H nmr: δ 1.38 (t,J=7.1 Hz,3H,CH_3), 4.45 (q,J=7.1 Hz,2H,CH_2), 7.25 - 7.50 (m,2H,ArH), 7.75 (br s,2H,NH_2), 8.00 - 8.25 (m,2H,ArH), 9.22 (s,1H,H-6).
Anal. Calcd for $C_{13}H_{12}FN_3O_3$ (277.25): C, 56.31; H, 4.36; N, 15.16. Found : C, 56.18; H, 4.36; N, 15.24.

2-Amino-3-ethoxycarbonyl-5-(p-chlorophenyl)pyrazine-1-oxide (7d)

Long bright yellow needles, mp. 198-200°; ^{1}H nmr: δ 1.37 (t,J=7.2 Hz,3H,CH_3), 4.44 (q,J=7.2 Hz,2H,CH_2), 7.58 (d,J=9 Hz,2H,ArH), 7.78 (br s,2H,NH_2), 8.08 (d,J=9 Hz,2H,ArH), 9.22 (s,1H,H-6).
Anal. Calcd for $C_{13}H_{12}ClN_3O_3$ (293.71): C, 53.16; H, 4.12; N, 14.31. Found : C, 52.94; H, 4.08; N, 14.32.

2-Amino-3-ethoxycarbonyl-5-(p-bromophenyl)pyrazine-1-oxide (7e)

After recrystallization from 1-propanol this compound was obtained as fine fluffy yellow needles, mp. 213-5°; ^{1}H nmr: δ 1.40 (t,J=7.5 Hz,3H,CH_3), 4.43 (q,J=7.5 Hz,2H,CH_2), 7.68 (d,J=9 Hz,2H,ArH), 7.77 (br s,2H,NH_2), 7.98 (d,J=9 Hz,2H,ArH), 9.18 (s,1H,H-6).
Anal. Calcd for $C_{13}H_{12}BrN_3O_3$ (338.17): C, 46.17; H, 3.58; N, 12.43. Found : C, 46.06; H, 3.58; N, 12.52.

2-Amino-3-ethoxycarbonyl-5-(p-cyanophenyl)pyrazine-1-oxide (7f)

The tan coloured precipitate was boiled up thrice with 250 ml of ethyl acetate and once with 350 ml of chloroform and filtered. The combined filtrates were evaporated and again boiled up in 300 ml of chloroform and filtered. After standing overnight at -20°, **7f** was obtained analytically pure as small yellow needles, mp. 235-8°; ^{1}H nmr: δ 1.38 (t,J=7.5 Hz,3H,CH_3), 4.43 (q,J=7.5 Hz,2H,CH_2), 7.88 (br s,2H,NH_2), 7.93 (d,J=9 Hz,2H,ArH), 8.24 (d,J=9 Hz,2H,ArH), 9.30 (s,1H,H-6).
Anal. Calcd for $C_{14}H_{12}N_4O_3$ (284.27): C, 59.15; H, 4.26; N, 19.71. Found : C, 58.89; H, 4.33; N, 19.48.

2-Amino-3-ethoxycarbonyl-5-(p-nitrophenyl)pyrazine-1-oxide (7g)

The orange precipitate was worked-up as described for **7f**. Recrystalliza-

tion from chloroform at -20° overnight gave **7g** as small orange-yellow needles, mp. 270-3°; ^{1}H nmr: δ 1.44 (t,J=7 Hz,3H,CH_3), 4.48 (q,J=7 Hz,2H,CH_2), 7.92 (br s,2H,NH_2), 8.20 - 8.25 (m,4H,ArH), 9.33 (s,1H,H-6).
Anal. Calcd for $C_{13}H_{12}N_4O_5$ (304.26): C, 51.31; H, 3.98; N, 18.42. Found : C, 51.30; H, 3.95; N, 18.65.

General procedure for the preparation of 2-amino-3-benzyloxycarbonyl-5-arylpyrazine-1-oxides

A mixture of the appropiate arylglyoxal-2-oxime (10 mmol), benzyl 2-amino-2-cyanoacetate methanesulfonic acid salt [37] (2.9 g, 10 mmol) and 15 ml of absolute methanol was stirred at room temperature during the time given in Table 3.1. After five minutes of stirring, a clear solution was formed and soon there after precipitation started. After the above indicated time the reaction mixture was diluted with 50 ml of ice-water and cooled for one hour. Then the precipitate was isolated by filtration and washed with about 10 ml of cold ethanol (4°) and 50 ml of cold ether (4°). However, in the case of **8b**, **8f** and **8g**, the precipitate was filtered off without dilution and washed with 50 ml of cold ether (4°) and dried. Recrystallization was performed from 1-propanol if not stated otherwise.

*2-Amino-3-benzyloxycarbonyl-5-(p-hydroxyphenyl)pyrazine-1-oxide (**8b**)*

Fine golden yellow needles, mp. 219-21°; ^{1}H nmr: δ 5.48 (s,2H,CH_2), 6.85 (d,J=9 Hz,2H,ArH), 7.35 - 7.75 (m,5H,ArH), 7.63 (br s,2H,NH_2), 7.85 (d,J=9 Hz,2H,ArH), 9.03 (s,1H,H-6), 9.76 (br s,1H,OH).
Anal. Calcd for $C_{18}H_{15}N_3O_4$ (337.32): C, 64.09; H, 4.48; N, 12.46. Found : C, 64.17; H, 4.47; N, 12.70.

*2-Amino-3-benzyloxycarbonyl-5-(p-fluorophenyl)pyrazine-1-oxide (**8c**)*

Fine bright yellow needles, mp. 175-6°; ^{1}H nmr: δ 5.47 (s,2H,CH_2), 7.23 - 7.72 (m,7H,ArH), 7.77 (br s,2H,NH_2), 7.95 - 8.20 (m,2H,ArH), 9.16 (s,1H,H-6).
Anal. Calcd for $C_{18}H_{14}FN_3O_3$ (339.32): C, 63.71; H, 4.16; N, 12.38. Found : C, 63.58; H, 4.13; N, 12.55.

*2-Amino-3-benzyloxycarbonyl-5-(p-chlorophenyl)pyrazine-1-oxide (**8d**)*

Bright yellow needles, mp. 181-3°; ^{1}H nmr: δ 5.50 (s,2H,CH_2), 7.36 - 7.76 (m,7H,ArH), 7.83 (br s,2H,NH_2), 8.08 (d,J=9 Hz,2H,ArH), 9.25 (s,1H,H-6).
Anal. Calcd for $C_{18}H_{14}ClN_3O_3$ (355.77): C, 60.76; H, 3.97; N, 11.81. Found : C, 60.52; H, 3.81; N, 11.73.

*2-Amino-3-benzyloxycarbonyl-5-(p-bromophenyl)pyrazine-1-oxide (**8e**)*

Fluffy bright yellow needles, mp. 176-9°; ^{1}H nmr: δ 5.50 (s,2H,CH_2), 7.32 - 7.72 (m,5H,ArH), 7.63 (d,J=9 Hz,2H,ArH), 7.75 (br s,2H,NH_2), 7.93 (d,J=9 Hz,2H,ArH), 9.19 (s,1H,H-6).

Anal. Calcd for $C_{18}H_{14}BrN_3O_3$ (400.23): C, 54.01; H, 3.53; N, 10.50. Found : C, 53.83; H, 3.51; N, 10.55.

*2-Amino-3-benzyloxycarbonyl-5-(p-cyanophenyl)pyrazine-1-oxide (**8f**)*

After recrystallization from 300 ml of chloroform and cooling overnight (-20°) this compound was obtained as yellow-orange needles, mp. 244-6°; ^{1}H nmr: δ 5.49 (s,2H,CH_2), 7.33 -7.66 (m,5H,ArH), 7.93 (d,J=9 Hz,2H,ArH), 7.96 (br s,2H,NH_2), 8.23 (d,J=9 Hz,2H,ArH), 9.33 (s,1H,H-6).

Anal. Calcd for $C_{19}H_{14}N_4O_3$ (346.33): C, 65.89; H, 4.07; N, 16.18. Found : C, 65.73; H, 3.99; N, 15.82.

*2-Amino-3-benzyloxycarbonyl-5-(p-nitrophenyl)pyrazine-1-oxide (**8g**)*

After recrystallization from dimethylformamide-1-propanol (3:2) this compound was obtained as long yellow needles, mp. 260-61°; ^{1}H nmr: δ 5.50 (s,2H,CH_2), 7.35 - 7.75 (m,5H,ArH), 8.00 (br s,2H,NH_2), 8.33 (br s,4H,ArH), 9.40 (s,1H,H-6).

Anal. Calcd for $C_{18}H_{14}N_4O_5$ (366.22): C, 59.04; H, 3.85; N, 15.30. Found : C, 58.85; H, 3.78; N, 15.28.

*General procedure for the preparation of 2-amino-3-carbamoyl-5-arylpyrazine-1-oxides **9***

The appropiate recrystallized 2-amino-3-ethoxycarbonyl-5-arylpyrazine-1-oxide or 2-amino-3-benzyloxycarbonyl-5-arylpyrazine-1-oxide (5 mmol) in 100 ml of liquid ammonia [1,12] was stirred during three hours under a drying tube. After evaporation of the ammonia, 20 ml of 2-propanol was added and the bright yellow precipitate was collected on a filter and washed with ether. Recrystallization was achieved from dimethylformamide.

*2-Amino-3-carbamoyl-5-(p-hydroxyphenyl)pyrazine-1-oxide (**9b**)*

Yield 96%, fine bright yellow needles, mp. 320° dec.; ^{1}H nmr: δ 6.85 (d,J=9 Hz,2H,ArH), 7.77 (br s,2H,NH_2), 7.82 and 8.32 (br s,1H,$CONH_2$), 7.98 (d,J=9 Hz,2H,ArH), 8.95 (s,1H,H-6), 9.74 (br s,1H,OH).

Anal. Calcd for $C_{11}H_{10}N_4O_3$ (246.22): C, 53.65; H, 4.09; N, 22.76. Found : C, 53.52; H, 4.11; N, 23.16.

*2-Amino-3-carbamoyl-5-(p-chlorophenyl)pyrazine-1-oxide (**9d**)*

Yield 92%, fine yellow needles, mp. 293-4°; ^{1}H nmr: δ 7.52 (d,J=9 Hz,2H,ArH), 7.97 (br s,2H,NH_2), 8.25 (d,J=9 Hz,2H,ArH), 7.97 and 8.47 (br s,1H,$CONH_2$), 9.15 (s,1H,H-6).

Anal. Calcd for $C_{11}H_9ClN_4O_2$ (264.67): C, 49.92; H, 3.43; N, 21.17. Found : C, 49.96; H, 3.41; N, 21.14.

*2-Amino-3-carbamoyl-5-(p-nitrophenyl)pyrazine-1-oxide (**9g**)*

Yield 95%, small yellow needles, mp. 324-6°; ^{1}H nmr: δ 7.95 (br s,1H,NH_2), 8.15 (br s,2H,NH_2 or $CONH_2$), 8.25 (d,J=9 Hz,2H,ArH), 8.52 (d,J=9 Hz,2H,ArH), 8.55 (br s,1H,$CONH_2$), 9.30 (s,1H,H-6).

Anal. Calcd for $C_{11}H_9N_5O_4$ (275.22): C, 48.00; H, 3.30; N, 25.45. Found : C, 47.84; H, 3.34; N, 25.06.

*Procedure for the ring closure of 2-amino-3-carbamoyl-5-arylpyrazine-1-oxides into 6-aryl-4(3H)-pteridinones-8-oxides **10***

A mixture of 2-amino-3-carbamoyl-5-arylpyrazine-1-oxide (2 mmol), 8 ml of triethylorthoformate and 8 ml of dimethylformamide was refluxed during 12 hours at 160°. After cooling to room temperature, the precipitate was isolated by filtration and washed with water, ethanol and ether. The compounds were finally recrystallized from dimethylsulfoxide-water.

*6-(p-Hydroxyphenyl)-4(3H)-pteridinone-8-oxide (**10b**)*

Yield 39%, yellow powder, mp. >350° dec.; ^{1}H nmr: δ 7.10 (d,J=9 Hz,2H,ArH), 8.17 (d,J=9Hz,2H,ArH), 8.33 (s,1H,H-2), 9.23 (s,1H,H-7).

Anal. Calcd for $C_{12}H_8N_4O_3$ (256.22): C, 56.25; H, 3.15; N, 21.87. Found : C, 56.09; H, 3.20; N, 21.61.

*6-(p-Chlorophenyl)-4(3H)-pteridinone-8-oxide (**10b**)*

Yield 47%, fine white needles, mp. >340° dec.; ^{1}H nmr: δ 7.69 (d,J=9 Hz,2H,ArH), 8.32 (d,J=9 Hz,2H,ArH), 8.39 (s,1H,H-2), 9.52 (s,1H,H-6).

Anal. Calcd for $C_{12}H_7ClN_4O_2*H_2O$ (292.68): C, 49.24; H, 3.10; N, 19.13. Found : C, 49.20; H, 3.27; N, 18.89.

Procedure for the preparation of 6-aryl-4(3H)-pteridinones 1

The appropiate 6-aryl-4(3*H*)-pteridinone-8-oxide(1 mmol) was dissolved in 10ml of 0.1 N sodium hydroxide and after the addition of sodium dithionite (90%, 0.95 g, 6 mmol) the mixture was refluxed during fifteen minutes. After

cooling the precipitate was filtered, again dissolved in a small amount of 0.1 N sodium hydroxide, filtered and acidified with a few drops of concentrated hydrochloric acid. Then the precipitate was dissolved in the minimal amount of 0.1 N sodium hydroxide and after the addition of 5 ml of 0.1 N potassium permanganate the mixture was stirred during ten minutes. The solution was acidified with gaseous sulfur dioxide, the precipitate was collected by filtration and recrystallized from aqueous dimethylsulfoxide.

*6-(p-Hydroxyphenyl)-4(3H)-pteridinone (**1b**)*

Yield 51%, yellow powder, mp. 340° dec.; ^{1}H nmr: δ 6.95 (d,J=9 Hz,2H,ArH), 8.13 (d,J=9 Hz,2H,ArH), 8.32 (s,1H,H-2), 9.52 (s,1H,H-7).
Anal. Calcd for $C_{12}H_8N_4O_3$ (256.22): C, 56.25; H, 3.15; N, 21.87. Found : C, 56.09; H, 3.20; N, 21.61.

*6-(p-Chlorophenyl)-4(3H)-pteridinone (**1d**)*

Yield 64%, white short needles, mp. 330-3°; ^{1}H nmr: δ 7.67 (d,J=7.5 Hz,2H,ArH), 8.30 (d,J=7.5 Hz,2H,ArH), 8.41 (s,1H,H-2), 9.66 (s,1H,H-7).
Anal. Calcd for $C_{12}H_7ClN_4O*\frac{1}{2}H_2O$ (267.68): C, 53.84; H, 3.01; N, 20.93. Found : C, 53.84; H, 2.65; N, 21.06.

Growth of cells and preparative scale conversion

Arthrobacter M-4 cells were grown as described previously. Both the immobilization and oxidation procedure were performed as earlier described [1,16,24].

For conversion of about 50 mg of **1b** or **1d** 30 units were used. The substrates were dissolved with the miminal amount of 4N of potassium hydroxide and diluted with potassium phosphate buffer pH=8.0 (I=0.01, 0.1 mM EDTA) to a volume of about 1000 ml. This solution was slowly passed with a velocity of 0.5 ml/min at room temperature through a column, containing the thoroughly washed cells entrapped in gelatine/glutaraldehyde. The conversion of substrates was followed by dilution of an aliquot in 0.1 N sodium hydroxide and measuring the UV-spectrum between 200-400 nm.

After 120 hours of reaction the column was run dry and the collected effluent was evaporated to a volume of about 100 ml and acidified with acetic acid. The percentage of conversion of the crude products was readily determined from integration of the appropiate signals of the nmr spectrum of the reaction mixture. The yields were 94% and 89% for **3b** and **3d**, respectively. Exact mass

data were identical to those of authentic specimen. Experimental values were 256.0604 and 274.0259 for **3b** and **3d**, respectively (theoretical values 256.0596 for $C_8H_{12}N_4O_3$ and 274.0258 for $C_{12}H_7{}^{35}ClN_4O_2$).

Kinetic assays

The preparation of the cell-free extract from frozen bacterial suspension and the assay for protein and activity were the same as previously described [1]. Each assay was performed at least in duplicate. For the xanthine oxidase from *Arthrobacter* M-4, one unit of enzyme activity is the amount of enzyme which oxidizes 1 μmol of 1-methylxanthine per min at 25°. The assay conditions were: 100 μM 1-methylxanthine in 50 mM potassium phosphate buffer, pH=7.25, including 0.1 mM EDTA with the reaction monitored at 292 nm ($\log \Delta\varepsilon = 4.09$), using oxygen as the final electron acceptor. The rate was determined from the initial slope of the absorbance *versus* time, representing the rate of appearance of the product.

A series of appropiate diluted solutions of inhibitor were mixed with 100 μM 1-methylxanthine in order to determine the inhibitory capacities of 7-aryl-4(3*H*)-pteridinones. Besides an uninhibited control, about five to seven concentrations were used which gave between 20% and 80% inhibition (*i.e.* for **2b** the range 1.51 to 15.1 μM while for **2g** as range 3.2 to 58.4 μM were used). The I_{50}-value for 7-aryl-4(3*H*)-pteridinones was calculated by plotting the logarithm of the inhibitor concentration *versus* the activity compared to an uninhibited control. The best fit was estimated by the method of linear least square and the mean of the concentration (of at least two determinations) at which 50% of the original activity was lost, is given in Table 3.2. The assay conditions were identical as those for 1-methylxanthine.

Kinetic parameters were estimated by the method of Naqui and Chance [46]. The assay mixture contained as buffer Tris-HCl (pH=8.0, I=0.05, 0.1 mM EDTA), as final electron acceptor oxygen and the substrate to be oxidized at appropiate concentrations (5 - 50 μM) in a final volume of 2 ml. Each assay was started by the addition of 0.05 or 0.1 ml of cell-free extract (approx. 2.5 mg protein/ml). The temperature of the assay mixture was maintained at 25°. The oxidation of 6-aryl-4(3*H*)-pteridinones was determined at a suitable wavelength using a DMS 100 spectrometer coupled with a DS 15 data station. The rate was determined from the time to exhaust half of the initial substrate concentration as a function of the initial substrate concentration. The appropiate wavelengths (λ in nm) and the corresponding mean molar differential absorption

coëfficiënts (log $\Delta\varepsilon$) are : **1a–3a**: 345 - 3.20, **1b–3b**: 353 - 3.34, **1d–3d**: 334 - 3.42, **1i–3i**: 345 - 3.28, **1j–3j**: 355 - 3.29.
Kinetic data were calculated from Hanes-Woolf plots [46,47].

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4 SYNTHESIS OF 3-ALKYL-6-PHENYL-4(3H)-PTERIDINONES AND THEIR 8-OXIDES; POTENTIAL SUBSTRATES OF XANTHINE OXIDASE [1]

4.1 INTRODUCTION

For several years there has been a current interest in our laboratory in the behaviour of 4(3*H*)-pteridinones, particularly of 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]. Similar behaviour was observed with *Arthrobacter* M-4 xanthine oxidase (AXO) towards 6-aryl-4(3*H*)-pteridinones [2d,3,4] where also exclusively oxidation at C-2 was observed. 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 reactions [5]. Similar results are also observed with the 8- and 9- phenyl-purines 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 also included 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.

4.2 SYNTHESIS OF 3-ALKYL-6-PHENYL-4(3H)-PTERIDINONES AND 8-OXIDES

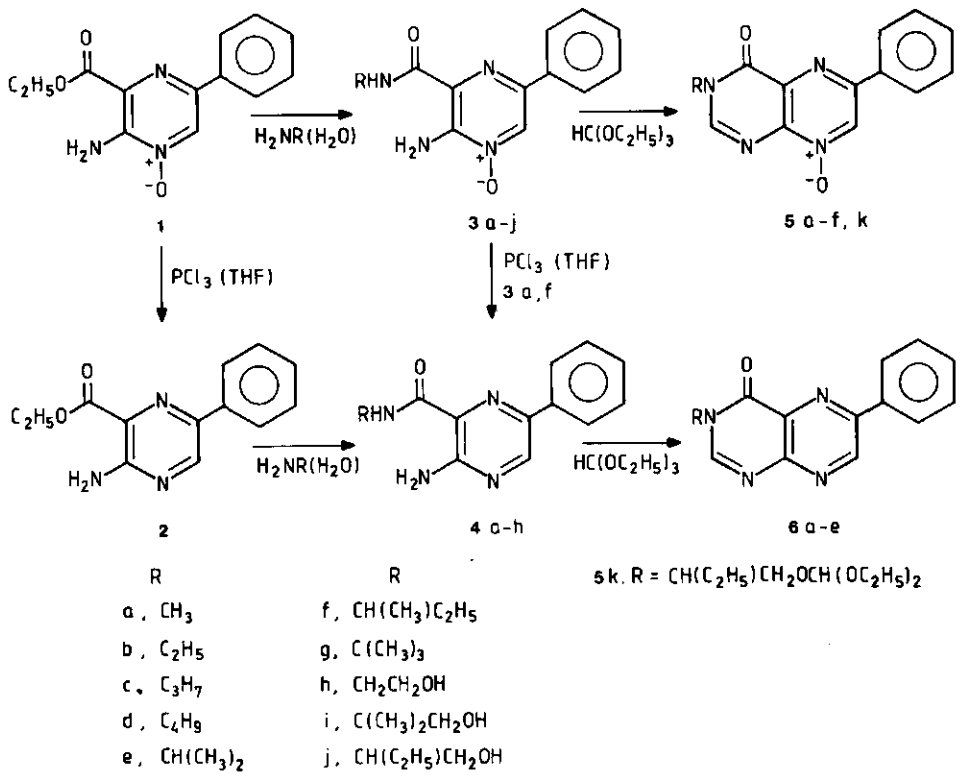
The key intermediate in our syntheses (Scheme 4.1) is 2-amino-3-ethoxycarbonyl-5-phenylpyrazine-1-oxide (**1**) [10]. The ethoxycarbonyl group in **1** reacts readily with amines to give the corresponding amides **3**. As amines we used methyl-, ethyl-, n-propyl-, n-butyl-, i-propyl-, s-butyl-, t-butyl-, 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 worked 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 occurred. 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]. With pure amides **3** the compounds **5** precipitated after cooling of the reaction mixture; the materials obtained, after washing with ethanol and ether, turned out to be analytically pure. Addition of a solvent like dimethylformamide [10] gave less satisfactory results.

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.

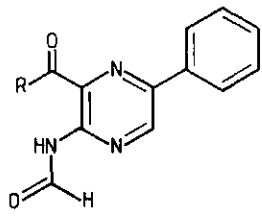
Removal of the N-oxide function from pteridine-8-oxides could be achieved by reduction with an aqueous sodium dithionite solution followed by treatment with potassium permanganate [4,10]. Yet, we did not use this procedure to prepare **6** but investigated an alternative route, *i.e.* cyclization of 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** was 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 ethoxygroup in **2** by an alkylamino group. This alkylamino-deethoxylation

reaction occurred readily with primary amines. Addition of water was again necessary to complete the reaction with secondary and tertiary amines (*e.g* no reaction occurred when t-butylamine was heated with **2** for three hours at 120°, but in the presence of water a yield of 92% was obtained).



Scheme 4.1

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 was formed. Since in the case of pyrazine-1-oxides, addition of acetic anhydride to the reaction might lead to rearrangements [12] no experiments were conducted in the presence of the last mentioned reagent.



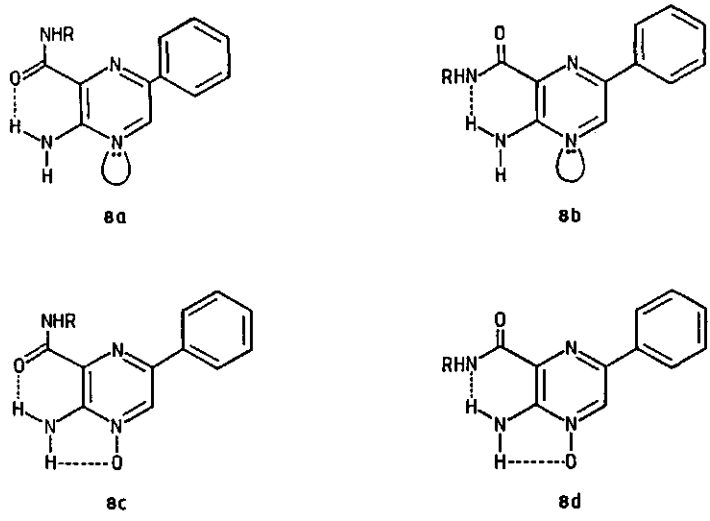
7

a, R = OC_2H_5

b, R = $NHCH(CH_3)C_2H_5$

Scheme 4.2

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 ^1H nmr spectra since attempts to purify them led to hydrolysis of the formyl group; **2** and **4f** were formed from **7a** and **7b** respectively.



8a 8b 8c 8d

*Figure 4.1 Possible configurations for compounds **3** and **4**: **8a** and **8b** are the conformations for **3**, whereas **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 carbonylfunction of the amide as depicted in Figure 4.1 (**8a, 8c**). In compounds **3** the 2-amino group is involved in an intramolecular H-bond with the N-oxide function. The forced conditions 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 allows ring closure.

Table 4.1 ^{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]	ΔH-6 [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	**3h**	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] ΔH-6 = δH-6 (**3**) - δH-6 (**4**).

[c] Resonance signals of the α-protons of 3-alkyl substituents.

Comparison of the chemical shifts of the ring protons H-6 in the compounds **3** and **4** (see Table 4.1) reveals that in the 1-oxides **3** H-6 is substantially deshielded (0.22 - 0.27 ppm). A similar deshielding is 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 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 cannot 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 is also found for other 5-substituted 2-amino-3-carbamoylpyrazines [4,18].

Table 4.2 ^{1}H *Nmr spectral data of 3-alkyl-6-phenyl-4(3H)-pteridinones (**6**) and their 8-oxides (5) (δ-values)* [a].

Alkyl substituent	Compound	H-7	N-CH [b]	Compound	H-7	N-CH [b]	ΔH-7 [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
$CH(CH_3)_2$	**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)				
$CH(C_2H_5)CH_2$-$OCH(OC_2H_5)_2$	**5k**	9.36(s)	4.70(1H,m)				

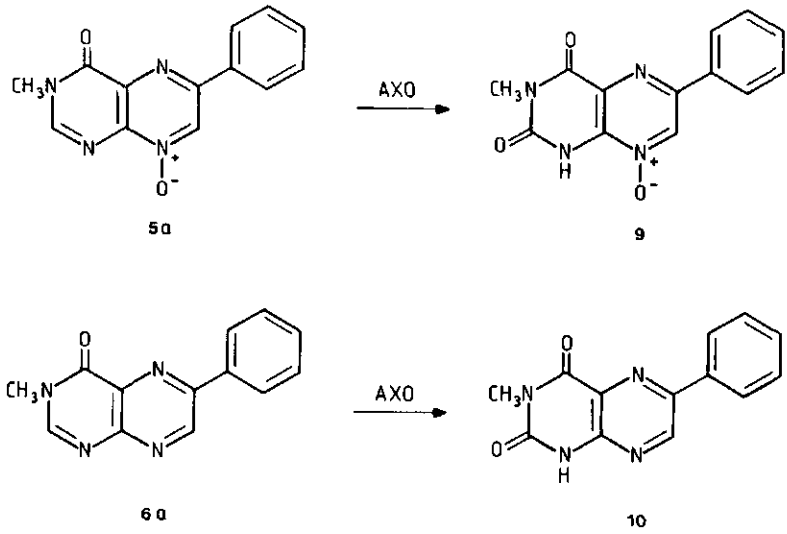
[a] Resonance signals of the 6-phenyl ring protons are over a range of 8.18 - 8.25 ppm (2H) and 7.46 - 7.57 ppm (3H).
[b] Resonance signals of α-protons of 3-alkyl substituents.
[c] ΔH-7 = δH-7 (**5**) - δ H-7 (**6**).

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 4.2). 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**.

4.3 ENZYMATIC KINETICS

Since the pH 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 pH. After a 100 μM 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 observed in the oxidation of 6-phenyl-4(3*H*)-pteridinone (**6**, R=H) [4]. At somewhat higher pH (7.5 and 8.0) hardly any conversion was observed, in agreement with reported earlier [3,22]. The oxidation at pH=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).



5a 9

6a 10

Scheme 4.3

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, which is 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 an N-oxide function at position 8 further decreases the rate of oxidation. When the methyl group in **5a** and **6a** is replaced by bulkier alkylgroups or by groups containing an 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 4.3 Inhibition parameters (I_{50}- and Ki-values) for the 3-alkyl-6-phenyl-4(3H)-pteridinones (**6**) at pH=7.25 using 100 μM 1-methylxanthine as substrate.* [a]

Compound	I_{50} [b]	Ki [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 ± 0.04 μmol/min.mg.

[b] In μmol/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 pH=7.25, using 1-methylxanthine as substrate [24]. The I_{50}-value obtained for the compounds **6a-e** are summarized in Table 4.3. 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 a 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.

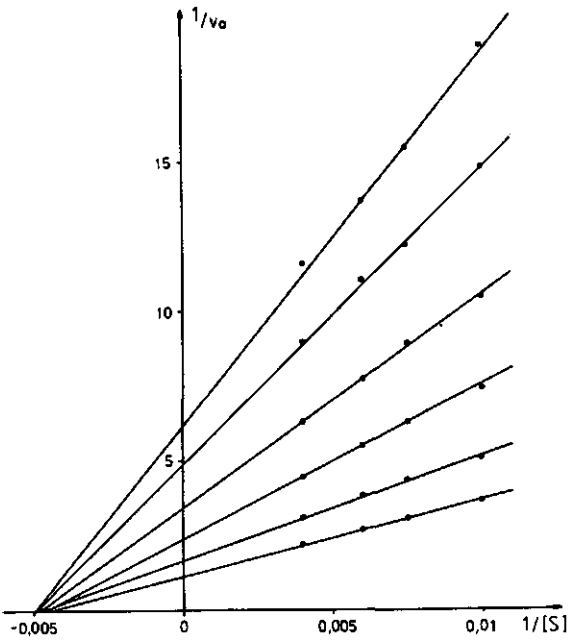


*Figure 4.2 Reciprocal plots for the oxidation of 100 μM 1-methylxanthine and five fixed concentrations of 3-methyl-6-phenyl-4(3H)-pteridinone (**6a**) by AXO at pH=7.25. The inhibitor concentrations used were (from bottom to top): 0, 24.9, 50.3, 59.9, 75.4 and 88.0 μM.*

We selected the compounds **6a** and **6b** to study the mode of inhibition of AXO in some detail. In Figure 4.2 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 reciprocal substrate concentration. This indicates that the Km value is not influenced by the inhibitor [26]. By replotting the data from Figure 4.2 (see Figure 4.3) 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.

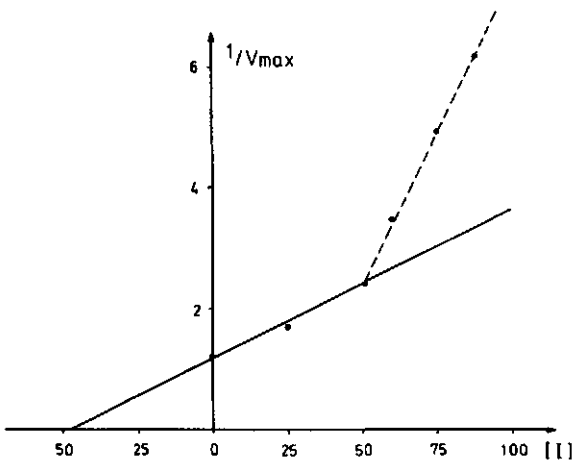


*Figure 4.3 A replot of the applied inhibitor concentrations of 3-methyl-6-phenyl-4(3H)-pteridinone (**6a**)* versus *the y-intercept (Vm^{-1}) of Figure 4.2.*

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 4.4; 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 4.4) an inhibition constant (Ki) of 19.6 μM is calculated. In contrary to the 3-methyl-derivative this compound is possibly not bounded in the vicinity of the active site of the enzyme but propably at another site. Apparently, the 3-ethyl derivative (partly) binds to the enzyme-substrate complex making a fraction of the offered AXO unavailable for oxidation of 1-methylxanthine.

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 provided 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.

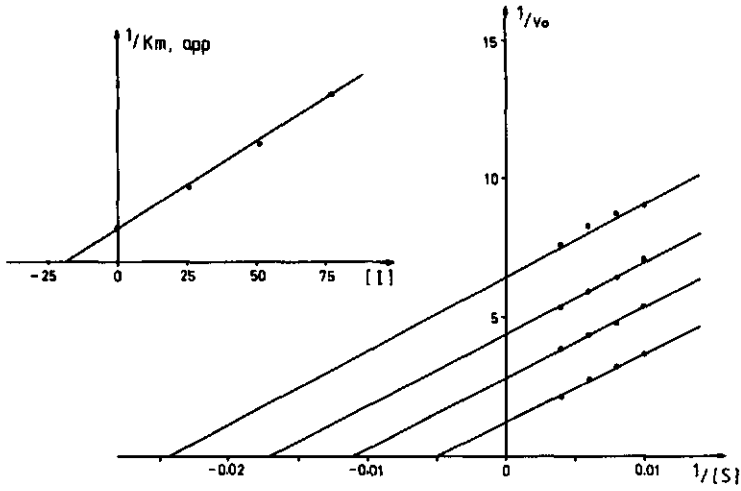


*Figure 4.4 Reciprocal plots for the oxidation of 100 µM 1-methylxanthine and three fixed concentrations of 3-ethyl-6-phenyl-4(3H)-pteridinone (**6b**) by AXO at pH=7.25. The inhibitor concentrations used were (from bottom to top): 0, 25.7, 51.5 and 77.2 µM. The insert shows a replot of the inhibitor concentration **6b** versus the corresponding apparent Km-value.*

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 questionable whether the small but distinct differences in electron density have an important impact on these rates.

4.4 EXPERIMENTAL SECTION

Melting points are uncorrected. The ^1H nmr spectra were recorded in deuterated dimethylsulfoxide 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 4.1 and 4.2 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 143-5° (lit. [10] 135-7°); ^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 mmole) in dry THF (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**)*

Yield 82%, yellow needles, mp 190-1°; 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**)*

Yield 91%, yellow needles, mp 170.0-171.5°; ^{1}H 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**)*

Yield 65%, yellow needles, mp 150-1°; ^{1}H 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**)*

Yield 96%, yellow needles, mp 122-3°; ^{1}H 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**)*

Yield 86%, yellow needles, mp 169-70°; ^{1}H 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**)*

Yield 80%, yellow needles, mp. 118-20°; ms: m/e 286 (M^+); ^{1}H 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**)*

Yield 77%, cream powder (from chloroform/methanol), mp. 214-5° dec; ^{1}H 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**)*

Yield 72%, yellow needles, mp 173-4°; ^{1}H nmr: δ 4.80 (exchangeable with

CD_3OD)(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-phenyl-pyrazine-1-oxide (**3i**)*

Yield 59%, fine yellow needles, mp 207-8°; ^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**)*

Yield 59%, cream powder, mp. 154-5°; 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**)*

Yield 85%, yellow needles, mp 130-1°.

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**)*

Yield 84%, 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**)*

Yield 91%, cream needles, mp 130-1°; ^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, 64.51; H, 6.20; N, 21.58.

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

Yield 92%, fine yellow needles, mp. 84-5°; ^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**)*

Yield 97%, cream coloured needles, mp. 125-6°; ^{1}H 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**)*

Yield 71%, cream needles, mp. 88-9°; ^{1}H 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**)*

Yield 92%, cream powder (from chloroform/methanol), mp 159-61° dec; ^{1}H 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**)*

Yield 76%, yellow needles, mp. 158-9°; ^{1}H 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(3H)-pteridinone-8-oxides (**5**) and of 2-amino-3-(alkylcarbamoyl)-5-phenylpyrazine (**4**) into 6-phenyl-3-alkyl-4(3H)-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 recrystallized from dimethyl sulfoxide-water (**5**) or chloroform-light petroleum ether (bp 40-60) (**6**).

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

This compound was obtained after 8 hours in a yield of 80% as a white

powder, mp 304-5° 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(3H)pteridinone-8-oxide (**5b**)*

This compound was obtained after 8 hours in a yield of 56% as a white powder, mp 288-90° 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(3H)pteridinone-8-oxide (**5c**)*

This compound was obtained after 6 hours in a yield of 34% as a white powder, mp. 247-9° dec;^{1}H nmr: δ (3H,t,CH_3), 1.76 (2H,m,CH_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(3H)pteridinone-8-oxide (**5d**)*

This compound was obtained after 6 hours in a yield of 94% as a white powder, mp 230-2° 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(3H)pteridinone-8-oxide (**5e**)*

This compound was obtained after 6 hours in a yield of 29% as a cream powder, mp 286-8° 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(3H)pteridinone-8-oxide (**5f**)*

This compound was obtained after 6 hours in a yield of 29% as a white powder, mp 231-2° 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(3H)pteridinone-8-oxide (**5k**)*

This compound was obtained after 16 hours in yield of 28% as a white

powder, mp 131-2°; ms: m/e 414.1907 (M^+) (Calcd. 414.1903), 398.1960 (M^+-16) (Calcd. 398.1954); ^1H 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(3H)pteridinone (**6a**)*

This compound was obtained after 6 hours in a yield of 78% as a cream powder, mp 235-6° dec.; ^1H 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(3H)pteridinone (**6b**)*

This compound was obtained after 6 hours in a yield of 49 % as a white powder, mp 215-6° dec; ms: m/e 252 (M^+); ^1H 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(3H)pteridinone (**6c**)*

This compound was obtained after 7 hours in a yield of 71% as white prisms, mp 133-4°; ^1H 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(3H)-pteridinone (**6d**)*

This compound was obtained after 8 hours in a yield of 32% as white prisms, mp 103-4°; ^1H 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(3H)-pteridinone (**6e**)*

This compound was obtained after 7 hours in a yield of 58% as white prisms, mp 160-1°; ms: m/e 266 (M^+); ^1H 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 CD_3OD 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 CD_3OD) 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 mM of potassium phosphate (pH=7.25, 0.1 mM EDTA) was used. Each assay was carried out at least in duplicate.

Stock solutions of all the compounds were prepared in 96% of ethanol whereby each 1 mM was first dissolved in one milliliter 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 μM 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 μM of substrate in 50 mM potassium phosphate buffer (pH=7.25, containing 0.1 mM EDTA) using oxygen as final electron acceptor. The reaction was monitored at 292 nm (log $\Delta\varepsilon$ = 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 μM of 1-methylxanthine was mixed with appropiate amounts of inhibitor, ranging from 25 to 250 μM. 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 μM of 1-methylxanthine and 24.9, 50.3, 59.9, 75.4 and 88.0 μM for compound **6a** (Figure 4.2) and using 100, 125, 167 and 250 μM of standard substrate and 25.7, 51.5 and 77.2 μM in the case of compound **6b** (Figure 4.3).

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5 INHIBITION OF BACTERIAL XANTHINE OXIDASE FROM ARTHROBACTER M-4 BY 5,6-DIAMINOURACIL

5.1 INTRODUCTION

Xanthine oxidase (xanthine: oxygen oxidoreductase, E.C. 1.1.3.22) is a complex molybdoflavoprotein widely distributed throughout the animal kingdom. Its major function is the oxidation of hypoxanthine and xanthine into uric acid in the process of purine degradation [1]. Xanthine oxidase is produced by only a few microorganisms. Most bacteria dissimilate xanthine by a NAD^+ specific dehydrogenase [2]. Xanthine oxidase found in *Arthrobacter* S-2 was isolated and its properties studied in detail [3]. Purification and properties of xanthine oxidase from *Enterobacter cloacae* has also been reported [4].

Immobilized xanthine oxidase has been used in studies on regiospecific oxidation of heterocyclic compounds [5,6]. Xanthine oxidase found in *Arthrobacter* X-4 is able to convert both 6-phenyl-4(3*H*)-pteridinone and 7-phenyl-4(3*H*)-pteridinone into the corresponding phenyllumazines [7], compounds which can not be prepared directly using mild reaction conditions. A laborious synthetic organic procedure is needed then. Because of its high phosphate requirement [3,5], *Arthrobacter* X-4 is disadvantageous when applied on a large scale. Therefore the xanthine oxidase producing *Arthrobacter* strain M-4 was isolated from garden soil. Differences in substrate specificity were observed between xanthine oxidases from bovine milk and *Arthrobacter* M-4 [3,5-8], indicating different properties of the active site of both enzymes.

The present study deals with the effects of 5,6-diaminouracil, allopurinol, bisalloxazine, and 8-phenylhypoxanthine (Fig. 5.1.) on purified xanthine oxidase from *Arthrobacter* M-4 and bovine milk. The two enzyme preparations responded very differently. It may therefore be concluded that the two xanthine oxidases differ with respect to their catalytic centra.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

5,6-Diaminouracil hemisulphate salt (**1a**) was purchased from Aldrich while 5,6-diaminouracil hydrochloride salt was synthesized according to the procedure given in the literature [9]. Xanthine (**1b**), allopurinol (**1c**) and bovine serum

albumine were purchased from Sigma. Chemicals used in media for bacterial growth were all from Merck. In order to prevent possible formation of bisalloxazine (**1d**), 5,6-diaminouracil was dissolved in 1 mM of kalium hydroxide shortly before use and was kept in the dark at 4 °. 1,3,6,8-Tetrahydro-2,4,5,7 (1*H*,3*H*,6*H*,8*H*)pyrimido[5,4-g]pteridinetetrone (bisalloxazine) was prepared by treatment of 5,6-diaminouracil with ferric chloride solution using the procedure described in the literature [10]. 8-Phenylhypoxanthine (**1e**) was prepared from 4,5-diamino-6(1*H*)-pyrimidinone and benzoic acid using the method developed for the synthesis of 2-arylimidazo[4,5-b]pyridines [11]. All compounds prepared were identified by their melting points, UV-spectra and elemental analyses.

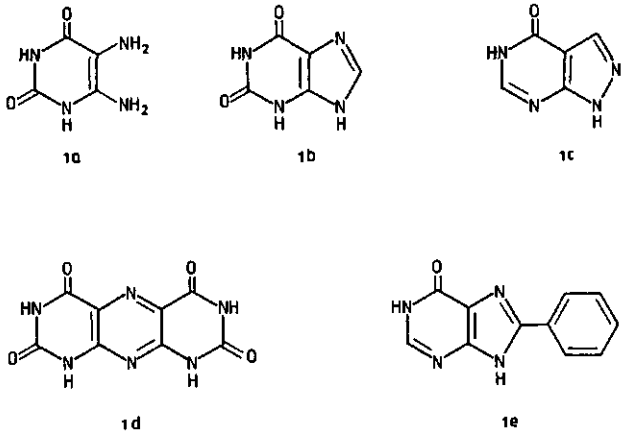

*Figure 5.1 Structural formula of 5,6-diaminouracil (**1a**), xanthine (**1b**), allopurinol (**1c**), bisalloxazine (**1d**) and 8-phenylhypoxanthine (**1e**).*

5.2.2 Bacterial strains

Arthrobacter strain M-4 was isolated from garden soil on a mineral salts medium containing xanthine as the sole source of carbon and nitrogen.

5.2.3 Growth of cells

Arthrobacter M-4 was grown at 30° and pH 7.2 in a 300 litre bioreactor (Bioengineering) in a growth medium containing per litre 5.0 g of glucose hydrate, 0.82 g of NH_4Cl, 2.0 g of K_2HPO_4, 3.0 g of KH_2PO_4, 0.1 g of $MgSO_4.7H_2O$, supplemented with vitamines and trace elements [12].

Glucose was depleted after about 24 hours following inoculation with a

preculture (1 litre) in a similar medium. At this point 1 mM of xanthine was added to the culture. After overnight induction of xanthine oxidase and depletion of xanthine the *Arthrobacter* M-4 culture was harvested at 10 000 g using a continuous centrifuge. The cells obtained in this way were used for further purification of xanthine oxidase.

5.2.4 Assay of xanthine oxidase

Rate measurements were carried out in a Beckmann recording spectrophotometer (Model 25). The conversion of xanthine into uric acid was followed at 293 nm. Milk xanthine oxidase was assayed at 25° with 0.1mM of xanthine as a substrate in 0.1 M of Tris-HCl buffer (pH=8.5, 0.1 mM EDTA) in a final volume of 1 ml. Xanthine oxidase from *Arthrobacter* M-4 was assayed at 25° with 0.1 mM of xanthine as a substrate in 0.1 M of potassium phosphate buffer (pH=7.2, 0.1 mM EDTA). The buffer and substrate solutions were oxygenated by flushing with air prior to start the reaction by addition of the enzyme solution.

Specific activities are expressed in units.mg^{-1} of protein. One unit of enzyme activity is defined as the amount of the enzyme that produces 1 µmol of uric acid per minute under standard conditions. Specific activities were calculated from initial reaction rates using molecular extinction coefficients for uric acid at 293 nm of 11.02 and 11.80 $mM^{-1}cm^{-1}$ at pH 7.2 and pH 8.5, respectively. In inhibition studies of xanthine oxidase the inhibitors were mixed with buffered substrate prior to starting the assay by addition of the enzyme solution.

Protein was determined according to a modified Lowry method [13], using bovine serum albumin as a standard.

5.2.5 Inhibition constants

The I_{50}-value (the concentration at which a compound shows a 50% inhibition of the initial reaction rate) was measured by graphic interpolation of a plot of reaction rate *versus* inhibitor concentration, at constant xanthine concentration (0.2 mM). A more accurate approach was possible if the inhibition was of the competitive type [14]. In that case Km and Ki were calculated by linear least square regression of a Lineweaver-Burk plot of the reaction rate *versus* xanthine concentration, at different inhibitor concentrations [14].

5.2.6 Purification of bovine milk xanthine oxidase

Xanthine oxidase from raw unpasteurized milk was purified according the to the method of Waud *et al.* [15]. The enzyme was purified to a specific activity

of 8.9 units.mg^{-1} of protein. The molecular relative mass (M_r) of the native enzyme (dimer) is 300 000 as determined by methods described in the literature [15].

5.2.7 Purification of xanthine oxidase from Arthrobacter *M-4*

The harvested cells were disrupted in a French press (Manteau). The crude extract had a specific activity of 0.32 units.mg^{-1} protein. It was treated with streptomycin sulphate to give a 1% (wt/vol) solution and was then centrifugated at 12 000 g for twenty minutes.

The supernatant was heated to 50° for ten minutes, centrifugated again and subjected to ammonium sulphate precipitation. The fraction precipitating between 30 and 53% saturation contained all xanthine oxidase activity and was collected by centrifugation at 10 000 g for ten minutes. The pellet was dissolved in a 10 mM potassium phosphate buffer (pH 7.0, 0.1 mM EDTA) and eluted over a Sepharose 6B column, which was previously equilibrated with the same buffer. The fractions containing the activity were brought on a DEAE Sepharose-CL-6B anion exchanger column previously equilibrated with 10 mM phosphate buffer (pH 7.0) and then eluted with a 0 - 1 M KCl gradient. Enzyme activity was found at approximately 0.4 M KCl; this fraction was dialyzed and concentrated by ultrafiltration (Amicon YM 30 filters).

As a final purification step part of the enzyme solution was brought on FPLC Mono Q HR 2/5 anion exchanger column (Pharmacia GP250), which was previously equilibrated with 20 mM Tris-HCl buffer (pH 7.5). Using a salt gradient of 0 - 1 M KCl, xanthine oxidase was eluted at 0.4 M KCl. This fraction was dialysed against 10 mM potassium phosphate buffer (pH 7.2, 0.1 mM EDTA).

The specific activity of this preparation was 50 units per mg of protein. The enzyme solution was stored at -20° until further use.

The molecular weight of xanthine oxidase from *Arthrobacter* M-4 was found to be 80 000 using both SDS-gel elektroforesis and FPLC. A Superose molecular sieve column (Pharmacia), was used which was previously equilibrated with a 50 mM solution of potassium phosphate buffer (pH 7.5) containing 0.15 M of sodium chloride.

5.3 RESULTS

5.3.1 Inhibition of bovine milk xanthine oxidase

Rates of inhibition of bovine milk xanthine oxidase using 8-phenylhypoxanthine, allopurinol, 5,6-diaminouracil and bisalloxazine as inhibitors were

measured at various inhibitor concentrations at pH 8.5. Figure 5.2 demonstrates that 8-phenylhypoxanthine is the most potent inhibitor of the enzyme, followed by allopurinol. The corresponding I_{50}-values are 0.5 μM for 8-phenylhypoxanthine and 8 μM for allopurinol.

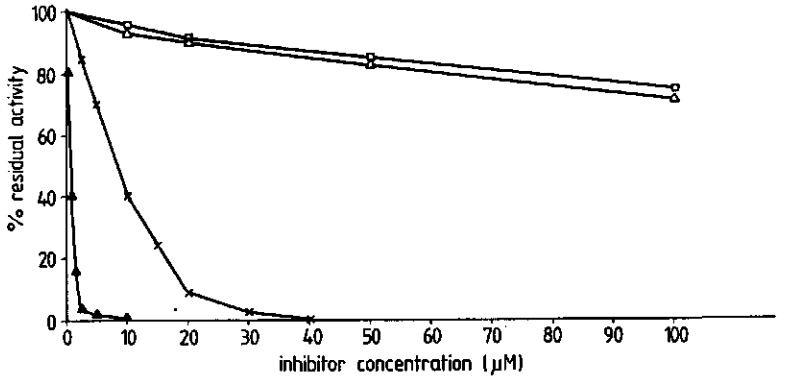

Figure 5.2 Plots showing inhibition rates of bovine milk xanthine oxidase by 8-phenylhypoxanthine (▲), allopurinol (X), 5,6-diaminouracil (△) and bisalloxazine (□) as a function of inhibitor concentration. Various concentrations of inhibitors were mixed with the buffered (pH=8.5) substrate xanthine (0.2 mM) prior to the addition of the enzyme.

Only a minor effect (30% inhibition at 100 μM) on the enzyme activity was observed for 5,6-diaminouracil and bisalloxazine (Fig.5.2). From the data presented in Figure 5.2 and assuming a further linear decrease in activity, the I_{50}-value seems to be at least 200 μM. The exact value could not be established due to solubility problems.

5.3.2 Inhibition of bacterial xanthine oxidase from Arthrobacter *M-4*

The I_{50}-value for 8-phenylhypoxanthine at pH 7.2 was deduced from the data in Figure 5.3 to be 2.0 μM. It is evident that this compound is less inhibitory for xanthine oxidase from *Arthrobacter* M-4 than for the milk enzyme. This is emphasized by the fact that complete inhibition required 40 μM of 8-phenylhypoxanthine, in contrast to the milk enzyme which required 10 μM.

The I_{50}-value of allopurinol was 12 μM. The inhibition is of the competitive nature as appears from Lineweaver-Burk plots (Figure 5.4). The inhibition constant was calculated as 8.4 μM. The Michaelis constant for xanthine is 109 μM. These results indicate that the inhibitory capacity of allopurinol is

less for xanthine oxidase from *Arthrobacter* M-4 than for that of milk.

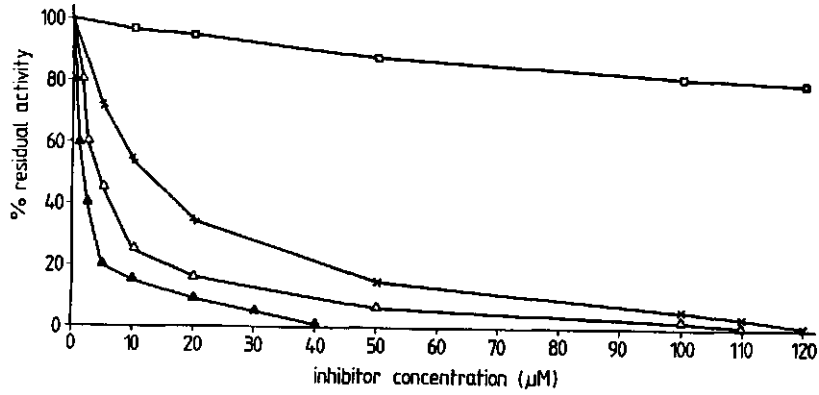

Figure 5.3 *Plots showing inhibition rates of xanthine oxidase from* Arthrobacter *M-4 by 8-phenylhypoxanthine (▲), allopurinol(X), 5,6-diaminouracil (△) and bisalloxazine (□) as a function of inhibitor concentration. Various concentrations of inhibitors were mixed with the buffered (pH=7.2) substrate xanthine (0.2 mM) prior to the addition of the enzyme.*

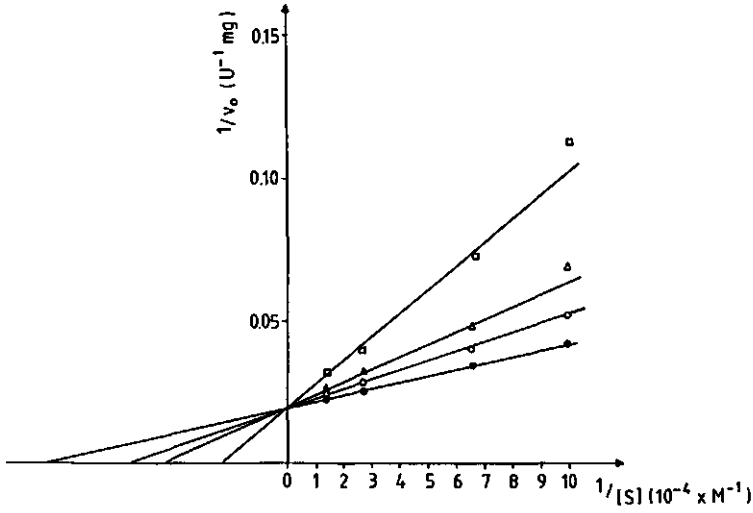

Figure 5.4 *Lineweaver-Burk plots for inhibition of xanthine oxidase from* Arthrobacter *M-4 by allopurinol: (●) = uninhibited control experiment; (○) = 5 μM; (△) = 10 μM; (□) = 20 μM.*

The most striking difference in inhibition was observed with 5,6-diaminouracil. The I_{50}-value for *Arthrobacter* M-4 was 4 μM, while for bovine milk xanthine oxidase an I_{50}-value of approximately 200 μ M was found. This result induced us to study the kinetics of the inhibition of *Arthrobacter* M-4 in more detail. The inhibition is competitive [14] with respect to xanthine (see Figure 5.5). The calculated inhibition constant is 0.98 μM. The Michaelis constant for xanthine was found to be 113 μM, corresponding fairly well with data presented by other workers: ranging from 110 μM [4] to 130 μM [3].

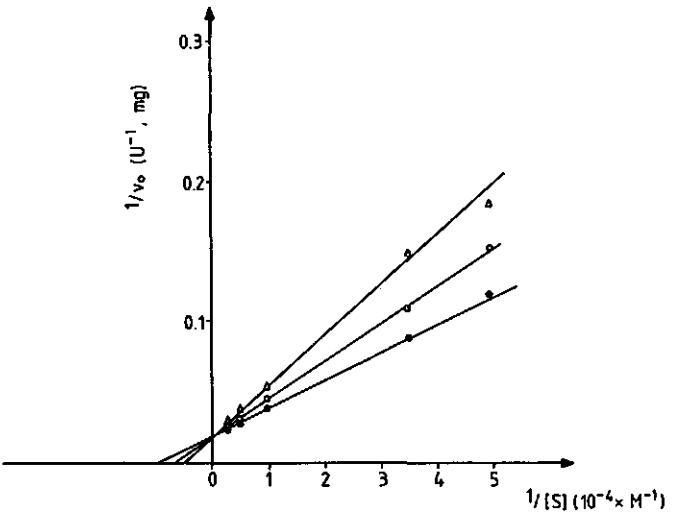

Figure 5.5 Lineweaver-Burk plots for inhibition of xanthine oxidase from Arthrobacter *M-4 by 5,6-diaminouracil: (●) = uninhibited control experiment; (○) = 0.5 μM; (Δ) = 1.0 μM.*

With respect to bisalloxazine the inhibition was negligibe therefore an I_{50}-value could not be estimated.

The Ki-values (table 5.1) show that allopurinol is about nine times weaker as competitive inhibitor of xanthine oxidase from *Arthrobacter* M-4 than 5,6-diaminouracil both with respect to xanthine.

5.4 DISCUSSION

The structural resemblance between 5,6-diaminouracil and xanthine induced us to investigate the possible inhibitory properties of this compound with the

two purified enzyme preparations.

5,6-Diaminouracil occurs in nature as its ribose phospate which is an intermediate in riboflavin synthesis [16]. It has also been reported to be an intermediate in the anaerobic degradative pathway of uric acid in *Clostridium purinolyticum* [17].

Table 5.1 Data on purified xanthine oxidase from bovine milk and Arthrobacter *M-4.*

	Enzyme source	
	Bovine milk	Arthrobacter M-4
Relative molecular mass (M_r)	300 000	80 000
pH optimum	8.5	7.2
Michaelis constant xanthine (μM)	28	111
Specific activity (units.mg^{-1})	8.9	50
I_{50} (8-phenylhypoxanthine, (μM)	0.5	2.0
Ki (allopurinol, μ M)	4.8	8.4
I_{50} (5,6-diaminouracil, μ M)	200	4
Ki (5,6-diaminouracil, μM)	n.d.[a]	2.0

[a] n.d. = not determined

5,6-Diaminouracil was found to inhibit xanthine oxidase from *Arthrobacter* M-4 but hardly affected bovine milk xanthine oxidase. Bisalloxazine, the dimeric condensation product from 5,6-diaminouracil, appeared to have only little effect on the enzyme from either source. 5,6-Diaminouracil and bisalloxazine were purified to a high very degree, therefore it can be concluded that the inhibitory properties are certainly not due to impurities, but to 5,6-diaminouracil itself.

Xanthine oxidase isolated from *Arthrobacter* S-2 by Woolfolk and Downard [3] was suggested to be *dimeric*, with approximate native and subunit molecular weights of 146 000 and 79 000 respectively. Although it is generally assumed that molybdohydroxylases are dimeric [18], our results suggest that xanthine oxidase from *Arthrobacter* M-4 is *monomeric* with M_r of 80 000 as determined both by SDS-gel electrophoresis and with a molecular sieve. Furthermore, xanthine oxidase from *Arthrobacter* S-2 prefers ferricyanide to oxygen as an electron acceptor [2,3]. The opposite was observed with *Arthrobacter* M-4 (not shown).

The molecular weight of xanthine oxidase from *Arthrobacter* M-4, like that of other bacterial sources [3,4] is significantly lower than that of bovine milk xanthine oxidase (purified according the method of Waud [15]). Moreover both enzymes differ with respect to their Michaelis constants, their specific activities in purified state and their pH optima [3,15,Table 5.1].

Xanthine oxidases from bovine milk and *Arthrobacter* M-4 have a broad substrate specificity [5,6,8]. This property makes the enzyme susceptible to inhibitors of a heterocyclic nature such as pteridines, purines and their aryl derivatives which in many cases are slowly convertible substrates [19-22]. Xanthine oxidase from milk and *Arthrobacter* M-4 differ also with respect to the oxidation rates of 6-phenyl- and 7-phenyl-(3*H*)-pteridinone [6-8,23]. In contrast to 7-phenyl-4(3*H*)-pteridinone, the 6-phenyl derivative is a poor substrate, which even acts as an effective inhibitor of bovine milk xanthine oxidase [23]. The enzyme from *Arthrobacter* M-4 on the other hand oxidizes 6-phenyl-4(3*H*)-pteridinone about twice as fast as the 7-phenyl derivative [7]. Evidence for the involvement of a hydrophobic region near the active site of bo vine milk xanthine oxidase is based on studies by Baker [20] and Robins [21] and is supported by studies with pteridines in which a phenyl group was introduced at C-6 [23]. 8-Phenylhypoxanthine is a potent inhibitor of xanthine oxidases from both sources (Table 5.1). However, the difference in I_{50}-values (0.50 μM and 2.0 μM), suggest that the region adjacent to the active site of bacterial xanthine oxidase is relatively less hydrophobic in nature.

This suggestion is supported by our results showing that 5,6-diaminouracil is a poor inhibitor of milk xanthine oxidase and a potent inhibitor of xanthine oxidase from *Arthrobacter* M-4. As the regio adjacent to the active center of milk xanthine oxidase is supposed to be more hydrophobic in nature than that of the bacterial enzyme, the latter is expected to be more susceptible to an an inhibitor with a polar structure like 5,6-diaminouracil.

Acknowledgements

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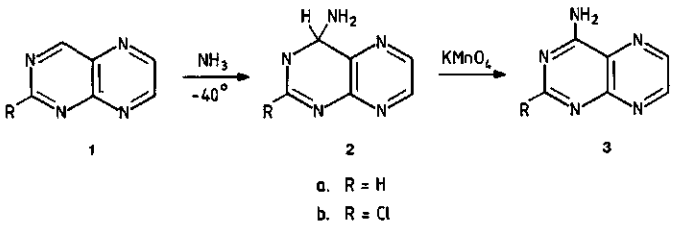
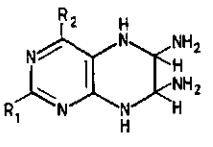
6 ON THE AMINATION OF PTERIDINES BY LIQUID AMMONIA-POTASSIUM PERMANGANATE

6.1 INTRODUCTION

It has previously been reported [1] that treatment of pteridine (**1a**) with potassium permanganate in liquid ammonia at -40° gave 4-aminopteridine (**3a**). This amination-oxidation procedure, when applied to 2-chloropteridine (**1b**) leads to an exclusive amination at C-4, yielding 4-amino-2-chloropteridine (**3b**). No amino-dechlorination at C-2 takes place. In both cases the intermediate species are the C-4 1:1 covalent σ-adducts 4-amino-3,4-dihydro-2-R-pteridines (**2a, b**) [2,3] (Scheme 6.1).





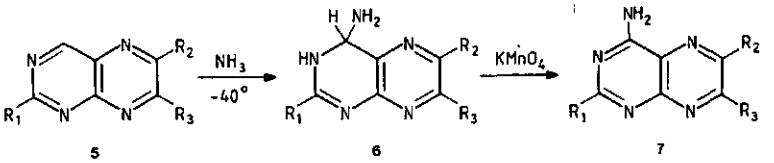
	R_1	R_2
a	H	H
b	OCH_3	H
c	SCH_3	H
d	Cl	CH_3
e	Cl	C_6H_5

Scheme 6.1

It was observed by means of ^1H and ^{13}C nmr spectroscopy that the regiospecificity of the addition is dependent on the temperature. At temperatures up to 25°, the addition of ammonia takes place to both C-6 and C-7, causing the formation of the 2:1 σ-adducts 6,7-diamino-5,6,7,8-tetrahydropteridines (**4**) [2,3].

6.2 RESULTS AND DISCUSSION

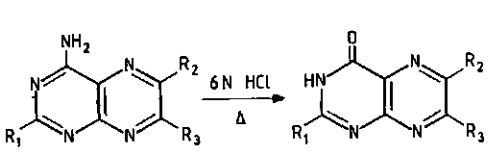
In an extension of this work we became interested in the behaviour of 7-, 6,7- and 2-substituted pteridines (**5a-5g**) towards potassium permanganate in liquid ammonia at -40° and at room temperature. Amination-oxidation of all these compounds at -40° according to the procedure reported [1] leads to introduction of one amino group. These reactions proceed with good yield and are summarized in Scheme 6.2.



	R_1	R_2	R_3
a	H	H	C_6H_5
b	H	H	p-$CH_3OC_6H_4$
c	H	H	CH_3
d	H	H	t-C_4H_9
e	H	C_6H_5	C_6H_5
f	H	CH_3	CH_3
g	C_6H_5	H	H

Scheme 6.2

The amino compounds obtained from the 7-aryl- and 7-alkylpteridines are all featuring the presence of two distinct low-field singlets in their respective ^1H nmr spectra. Since these data do not allow an unequivocal assignment of the position of the amino group *i.e.* position 2, 4 or 6, we converted the amino compounds **7a, 7d** into their respective 4(3*H*)-pteridinones.



Scheme 6.3

By heating with 6N hydrochloric acid for 15 minutes the 4(3*H*)-pteridinones **8a**

Table 6.1 Nmr spectral data of the compounds described (δ-values)

Pteridine	H2	H4	H6	H7	Solvent [c]
unsubstituted	9.66 (s)	9.82 (s)	9.13 (d) [a]	9.32 (d) [a]	A
7-phenyl-(**5a**)	9.62 (s) [a]	9.74 (s)	9.60 (s) [a]	–	A
4-amino-3,4-dihydro-7-phenyl-(**6a**)	7.68 (s)	5.58 (s)	8.88 (s)	–	B
4-amino-7-phenyl-(**7a**)	8.60 (s)	–	9.48 (s)	–	C
7-*p*-methoxyphenyl-(**5b**)	9.60 (s)	9.70 (s)	9.52 (s)	–	A
4-amino-3,4-dihydro-7-*p*-methoxyphenyl (**6b**)	7.64 (s)	5.50 (s)	8.82 (s)	–	B
4-amino-7-*p*-methoxyphenyl-(**7b**)	8.51 (s)	–	9.37 (s)	–	C
7-methyl- (**5c**)	9.60 (s)	9.71 (s)	8.97 (s)	–	A
4-amino-3,4-dihydro-7-methyl- (**6c**)	7.58 (s)	5.49 (s)	8.18 (s)	–	B
4-amino-7-methyl-(**7c**)	8.57 (s)	–	8.80 (s)	–	C
7-*t*-butyl-(**5d**)	9.49 (s)	9.59 (s)	9.12 (s)	–	A
4-amino-3,4-dihydro-7-*t*-butyl- (**6d**)	7.57 (s)	5.52 (s)	8.42 (s)	–	B
4-amino-7-*t*-butyl- (**7d**)	8.54 (s)	–	9.00 (s)	–	C
6,7-diphenyl-(**5e**) [b]	9.60 (s)	9.78 (s)	–	–	A
4-amino-6,7-diphenyl-(**7e**)	8.60 (s)	–	–	–	C
6,7-dimethyl- (**5f**)	9.54 (s)	9.63 (s)	–	–	A
4-amino-3,4-dihydro-6,7-dimethyl (**6f**)	7.48 (s)	5.43 (s)	–	–	B
4-amino-6,7-dimethyl (**7f**)	8.44 (s)	–	–	–	C
2-phenyl-(**5g**)	–	9.82 (s)	9.00 (d) [a]	9.23 (d) [a]	A
1:1 σ-adduct(**6g**)		5.60 (br)	8.05 (d) [a]	8.12 (d) [a]	B
2:1 σ-adduct		7.78 (s)	4.12 (d) [a]	4.25 (d) [a]	B
4-amino-2-phenyl-(**7g**)	–	–	8.81 (d)	9.11 (d)	C

[a] Resonance signals may be interchanged.

[b] Due to its high insolubility in liquid ammonia no resonance signals have been recorded at -40° as well as when the solution is allowed to be at room temperature.

[c] A: Deuteriochloroform; B: Liquid ammonia; C: Dimethylsulfoxide-d_6.

and **8d** were obtained and found to be identical with the corresponding 4(3*H*)-pteridinones, synthesized independently [4,5]. It indicated that in the amination-oxidation reaction the amino group is substituted at position 4 of the pyrimidine ring. The structure of compound **7a** was confirmed by comparing its physical and chemical properties with those of the compound obtained by the condensation of 4,5,6-triaminopyrimidine sulfate with phenylglyoxal [6].

The structure of the amino compounds obtained from the 6,7-disubstituted pteridines was also based on ^{1}H nmr data (see Table 6.1) and on the conversion of one of the amino compounds *i.e.* **7e** into the known 4(3*H*)-pteridinone **8e** [7,8].

The 4-amino structure of the product obtained from **5g** was proved by its ^{1}H nmr spectrum showing the two *doublets* (J = 3 Hz) of the hydrogens at C-6 and C-7.

Also ^{13}C nmr data were in accordance with the assigned structures. Compound **7d** (in deuterated dimethylsulfoxide) gives among others two ^{13}C-resonance signals at 159.2 (C-2) and 141.8 (C-6) ppm. They are associated with bond ^{13}C-^{1}H coupling constants of 196 and 185 Hz, respectively. In the case of compound **7e** the signal at 159.4 (C-2) ppm has the coupling constant ^{1}J (CH) = 198 Hz. In ^{13}C nmr spectra of both compounds **7d** and **7e** a downfield shift (about 9 ppm) for C-10 is observed, being also found in the ^{13}C nmr spectrum of 4-aminopyrimidine [9].

To investigate whether the reactions described above proceed by the intermediary of 4-amino-7R(6,7-R or 2R)-3,4-dihydropteridines, ^{1}H nmr spectra of compounds **5a** - **5g** were measured in liquid ammonia at -40° (in the case of **5g** also at -60°). It was observed (Table 6.1) that in this solvent the absorption of hydrogen atom in position 4 of compounds **5a** - **5d**, and **5f** was found to be highly upfield shifted ($\Delta\delta \sim 4$ ppm) compared to the one found for H-4 in solutions of **5a** - **5d** and **5f** in deuteriochloroform. This upfield shift was attributed to the formation of the -adducts **6a** - **6d**, **6f** involving the change of hybridization of C-4 from sp^2 (in **5a** - **5d**, **5f**) to sp^3 (in **6a** - **6d**, **6f**). These results strongly indicate the involvement of these σ-adducts in the amination-oxidation reaction.

Under above mentioned conditions the ^{1}H nmr spectrum of compound **5e** only showed a low noise base line probably because of its sparing solubility in liquid ammonia. Since in our amination-oxidation experiment a large excess of ammonia was used, we were still able to obtain the 4-aminoderivative **7e** in good yield.

The ^{1}H nmr spectrum of the compound **5g**, dissolved in liquid ammonia (-60°)

showed besides the signals characteristic of the 1:1 σ-adduct (**6g**) also those of the 2:1 σ-adduct **4** (R = C_6H_5, R_1 = H). At -40 ° only traces of 1:1 σ-adduct were observed. As the amination-oxidation reaction was carried out in the presence of potassium permanganate, which oxidizes the 1:1 adduct faster than the 2:1 adduct the equilibrium probably shifts in favour of 1:1 adduct yielding mainly 4-aminoderivative **7g**. Only traces of the 6,7-diamino compound were detected by mass spectrometry in the reaction product (The same effect was observed with pteridine itself).

As was already indicated before in pteridine the regiospecificity of the addition is dependent of the temperature. Therefore we also measured the ^{1}H nmr spectra of solutions of the pteridine derivatives **5** in liquid ammonia after these solutions have been allowed to come to room temperature. We observed nearly no change in the ^{1}H nmr spectra of compounds **5a - 5d, 5f,** proving that the addition at C-4 is also occurring at higher temperature. Apparently due to the presence of an aryl or alkyl substituent at 7, addition at C-6 is prevented; this means that in the pteridines **5** the kinetic and thermodynamically controlled addition favours the same position 4. A solution of **5e** at +20 ° did not show any signals; in the spectrum of **5g** only the presence of 2:1 σ-adduct was recorded (see Table 6.1).

To determine the stability of the σ-adducts ^{1}H nmr spectra of compounds **5a - 5g** were measured after 5, 45, 60 minutes or even after standing overnight at room temperature. Under these conditions only the σ-adducts of 7-phenyl- and 7-t-butylpteridines were found to be fully stable. The adduct of 7-*p*-methoxyphenylpteridine slowly decomposed and gave a yellow precipitate. The solution of 7-methylpteridine turned to dark blue and black after 5 and 60 minutes, respectively. Similarly, the solution of 6,7-dimethylpteridine coloured dark blue after 15 minutes. Even after standing overnight no ^{1}H nmr resonance signals of **5e** were observed. The 2:1 σ-adduct of 2-phenylpteridine is stable after standing 30 minutes at room temperature but decomposes during staying overnight.

6.3 EXPERIMENTAL SECTION

Melting points are uncorrected. The IR spectra were obtained as potassium bromide pellets using spectrometer A-100 (Jasco). The ^{1}H nmr spectra were recorded on Hitachi Perkin-Elmer R-24B (60 MHz) and Varian EM-390 (90 MHz) spectrometers with TMS as internal standard. The mass spectra were obtained on AEJ MS-902, equipped with a VG-ZAB console. For the column chromatography

silicagel 60 (70-230 mesh ASTM) Merck was used. The data of ^{1}H nmr spectra are shown in Table 6.1.

General procedure for the amination of pteridines **5**.

A. At low temperature (-40°).

To a stirred solution of liquid ammonia (20-25 ml) containing 316 mg (2 mmoles) of potassium permanganate the corresponding pteridine (2 mmoles) was added in one portion. The stirring was continued for 4 hours (compounds **5a** - **5d**, **5g**) or 6 hours (compounds **5e**, **5f**), at the temperature of about -40°. Then the ammonia was evaporated at room temperature and to the residue methanol (50 ml) was added. The mixture was allowed to stand overnight. Brown precipitate (manganese dioxide) and separated substances (in the case of the compounds **7a**, **7b**, **7e**, **7f**) were filtered by suction and washed with methanol. The residue on the filter was exhaustively extracted with boiling methanol (compounds **7a**, **7b**) or boiling chloroform (compounds **7e**, **7f**). To the combined methanol (or methanol-chloroform) solutions silica gel (1-2 g) was added and the solvent was evaporated under reduced pressure. The residue was subjected to column chromatography on silica gel for purification (eluent: methanol/chloroform 1:9 (compounds **7c**, **7d**), or methanol/ chloroform 1:19 (compounds **7a**, **7g**) or chloroform and methanol/ chloroform 1:19 (compounds **7b**, **7e**, **7f**).

B. At room temperature (20°).

A mixture of 0,5 mmole of the pteridine **5a** - **5d** and about 5 ml of liquid ammonia was sealed in a glass tube filled with nitrogen and maintained at room temperature for 1 hour (compounds **5a**, **5c**, **5d**) or 2 hours (compound **5b**). Under these conditions 7-methylpteridine gave a blue and then a black solution, 7-*t*-butylpteridine turned into a pale yellow solution and 7-phenylpteridine after dissolving first yielded a slight yellow precipitate. In the case of 7-*p*-methoxyphenylpteridine a yellow precipitate was observed from the beginning of the reaction. Thereupon 79 mg (0,5 mmole) of potassium permanganate was introduced to the reaction mixture after having been cooled to -40°, and stirred. After 10 minutes ammonia was allowed to evaporate and to a dark residue methanol (20 ml) was added; the mixture was left overnight. Then the brown precipitate was filtered by suction and the solvent was evaporated under reduced pressure. In the case of the compounds **7a**, **7b**, the residue on the filter was exhaustively extracted with boiling methanol.

The obtained products were purified by column chromatography using the same eluents as described in section A. In all cases the same substances were

obtained as by the procedure mentioned in section A.

4-Amino-7-phenylpteridine (7a)

Yield 90% (method A), 60%, besides 30% of the starting material (method B). Cream-coloured crystals, mp. 284-286° (from dimethylsulfoxide/water), lit. [6], mp. 265-267°, ms: m/e 223,0861 (M^+) (Calcd. 223,0858), ir: 3360, 3300 cm^{-1} (NH_2). The ir and ^1H nmr spectra of this compound were identical with those of an authentic sample prepared by the condensation of 4,5,6-triaminopyrimidine sulfate with phenylglyoxal according to the prescription given in [6]. After several crystallizations from aqueous dimethylsulfoxide the obtained substance melted at 284-5°.

*4-Amino-7-*p*-methoxyphenylpteridine (7b)*

Yield 61%, besides 24% of the starting material (method A), and 10%, besides 80% of the starting pteridine (method B). Yellow crystals, mp. 297-300° (dec.) (from dimethylsulfoxide), ms: m/e 253 (M^+).
Anal. Calcd for $C_{13}H_{11}N_5O$ (253,26): C,61.65; H,4.38. Found: C,61.85; H,4.10.

4-Amino-7-methylpteridine (7c)

Yield 88% (method A), 10% (method B). Colourless crystals mp. 246-248° (from chloroform/ether), ms: m/e 161 (M^+).
Anal. Calcd for $C_7H_7N_5$ (161.17): C,52.16; H,4.38. Found: C,51.87; H,4.09.

4-Amino-7-t-butylpteridine (7d)

Yield 92% (method A), 90% (method B). Colourless crystals mp. 265-266° (from chloroform/light petroleum bp. 80-100°), ms: m/e 203 (M^+).
Anal. Calcd for $C_{10}H_{13}N_5$ (203.24): C,59.09; H,6.45. Found: C,58.90; H,6.05.

4-Amino-6,7-diphenylpteridine (7e)

Yield 70% (method A). Light yellow needles mp. 173-5° after solidifying mp. 205-207° (from aqueous acetone), lit. [10] mp. 175°, ms:m/e 299 (M^+).

4-Amino-6,7-dimethylpteridine (7f)

Yield 47% (method A). Colourless crystals mp. 295° (dec.) (from water), lit. [11,12], mp. 295° (dec.), ms: m/e 175 (M^+).

4-Amino-2-phenylpteridine (7g)

Yield 50% (method A). Colourless crystals mp. 250-251° (from ethanol),

lit. [13] mp. 239-240°, ms: m/e 223 (M^+).

*7-Phenyl-4(3H)-pteridinone (**8a**)*

A solution of 100 mg (0.448 mmol) of the compound **7a** in 5 ml of 6N hydrochloric acid was heated under reflux for 15 minutes. After cooling the separated crystals were filtered off, washed with water until acidic reaction disappeared and dried, 72 mg (72% yield) of colourless crystals were obtained, mp. > 300° (dec.) (from aqueous dimethylformamide), lit. [4] mp. 295° (dec.); ms: m/e 224 (M^+), ir: 1720 cm^{-1} (CO). The ir and ^1H nmr spectra of this compound were identical with those of an authentic sample prepared by the oxidation of 7-phenylpteridine with m-chloroperbenzoic acid [4].

*7-t-Butyl-4(3H)-pteridinone (**8d**)*

This compound was obtained in the same way as described above for 7-phenyl-4(3*H*)-pteridinone, starting from 100 mg (0.49 mmole) of 4-amino-7-t-butylpteridine yielded 62 mg (62%) of **8d** as colourless crystals, mp.> 300° (dec.) (after reprecipitation from a dilute sodium hydroxide solution by acetic acid), lit. [5] mp. was not given, ms: m/e 204 (M^+), ir: 1715 (CO).
The ir spectrum of this substance was identical with that of 7-t-butyl-4(3*H*)-pteridinone obtained by the condensation of 4,5-diamino-6(1*H*)-pyrimidinone with t-butylglyoxal at pH 7.5 [5].

*6,7-Diphenyl-4(3H)-pteridinone (**8e**)*

This compound was obtained in the same way as the 4(3*H*)-pteridinone derivatives **8a** and **8d** presented above; 60 mg (0.2 mmole) of the compound **7e** gave after reprecipitation from a dilute solution of sodium hydroxide by acetic acid 31 mg (52%) of colourless crystals, mp. 308-310° (dec.), lit. [7,8] mp. 297-298°, 295°, respectively, ms: m/e 300 (M^+), ir: 1698 cm^{-1} (CO); ^1H nmr (dmso-d_6): δ 7.45 (m,10H,ArH), 8.45 (s,1H,H-2). Ir and ^1H nmr spectra of this compound were identical with those of the substance obtained by the condensation of 4,5-diamino-6(1*H*)-pyrimidinone with benzil [7].

Acknowledgement

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7 COMPARISON OF THE OXIDATION OF HETEROAROMATICS BY BOVINE MILK XANTHINE OXIDASE AND BY XANTHINE OXIDASE FROM ARTHROBACTER M-4

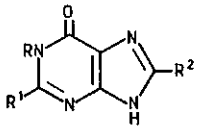
7.1 INTRODUCTION

The oxidation of the π-deficient heteroaromatics like purines and pteridines by chemical methods is usually difficult: the rings prevent electron donation to the oxidizing agent. Since 1975 there is ongoing interest in our laboratory for hydroxy functionalization of heteroaromatics using (immobilized) enzymes and bacterial cells [1,2]. Bovine milk xanthine oxidase (MXO) is an intensively studied enzyme [3-6] which effectively catalyses the oxidation of electron-deficient heteroarenes. Also xanthine oxidase present in *Arthrobacter* cells (AXO) [7] is an effective catalyst; it has a specific activity of about fifty times [8] that of the milk enzyme. Interestingly AXO has a different, but a more narrow substrate specificity than the milk enzyme [1,2,7,8]. Unlike MXO, AXO shows substrate activation [8] rather than substrate inhibition. Since AXO, in contrast to MXO, is less well studied we investigated the possibility of applying (immobilized) bacterial *Arthrobacter* M-4 cells, containing xanthine oxidase for regiospecific oxidation. In addition we tried to elucidate which oxidation site-determining factors are important for bacterial xanthine oxidase from *Arthrobacter* M-4. In the following sections we briefly describe the results of oxidation of several classes of compounds *i.e.* purines [9], pteridines [10] and their lineair-benzo derivatives [11], with AXO and MXO and compare their reactivities, expressed in product formation, Vm and Km values.

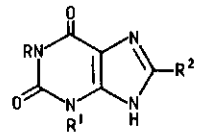
7.2 HYPOXANTHINES AND XANTHINES

Only a few reports are available on the oxidation of simple purines by bacterial xanthine oxidases [7,8,12]. Most of them concern the relative velocity of oxidation compared with that of hypoxanthine (**1**) or xanthine (**5**), the natural substrates for this enzyme. We investigated the oxidation of some derivatives of hypoxanthine and xanthine *i.e.* 2-methylhypoxanthine (**2**), 8-methylhypoxanthine (**3**), 1-methyl-2(methylthio)hypoxanthine (**4**), 1-methylxanthine (**6**) and 3-methylxanthine (**7**) using AXO as oxidant, and compared the results with those obtained by oxidation with MXO. We observed that the favoured position of attack by xanthine oxidase on substrates with more than one

oxidazible position sometimes varies dramatically from one enzyme source to another. Compound **7** is not oxidized by either AXO or MXO, while **6** is a good substrate for both enzymes; **3** is a good substrate for the milk enzyme, but is not attacked by the bacterial one; 2-methylhypoxanthine (**2**) is oxidized at C-8 by both AXO and MXO, but in a rather low rate by the latter. The rate of the oxidation is also strongly determined by the enzyme source (see Tables 7.1 and 7.2). Unexpectedly 1-methyl-2-(methylthio)hypoxanthine (**4**) is slowly converted by AXO, but not at all by milk xanthine oxidase.



	R	R^1	R^2	position of attack MXO	AXO
1 :	H	H	H	2	2
2 :	H	CH_3	H	8	8
3 :	H	H	CH_3	2	nr
4 :	CH_3	SCH_3	H	nr	8



5 :	H	H	H	8	8
6 :	CH_3	H	H	8	8
7 :	H	CH_3	H	nr	nr

nr = no reaction

Scheme 7.1

Introduction of a methyl group in the various positions of hypoxanthine or xanthine not only considerably influences the rate of oxidation by MXO as well as AXO, but also the Km values. In reactions with AXO it was found that introduction of a methyl group at C-2 of hypoxanthine increases the Km value about five fold and that introduction of a methyl group at N-1 of xanthine increases the Km seven fold (see Table 7.1).

These results indicate that introduction of a methyl group in substrates as hypoxanthine and xanthine always lowers the affinity of the substrate towards AXO considerably. For MXO the differences are even more pronounced: at pH=8.0 a twenty seven fold increase of Km is found between **5** and **6** (see Table 7.2).

Table 7.1 Kinetic parameters of substrate oxidized by AXO in potassium phosphate buffer at pH=7.2

Compound	1	2	3	4	5	6	7	8
Km (μM)	50.7	260	NR [b]	ND [c]	109	750	NR	108
Vm [a]	13	31	NR	slow	100	480	NR	21

[a] Vm is expressed as percent of Vm of xanthine.
[b] NR = No Reaction.
[c] ND = Not Determined.

Table 7.2 Kinetic parameters of substrates oxidized by MXO in potassium phosphate buffer at pH=8.0

Compound	1	2	3	4	5	6	7	8
Km (μM)	3.3	ND [c]	11.1	NR [b]	5.3	143	NR	5.6
Vm [a]	108	0.03	94	NR	100	141	NR	24

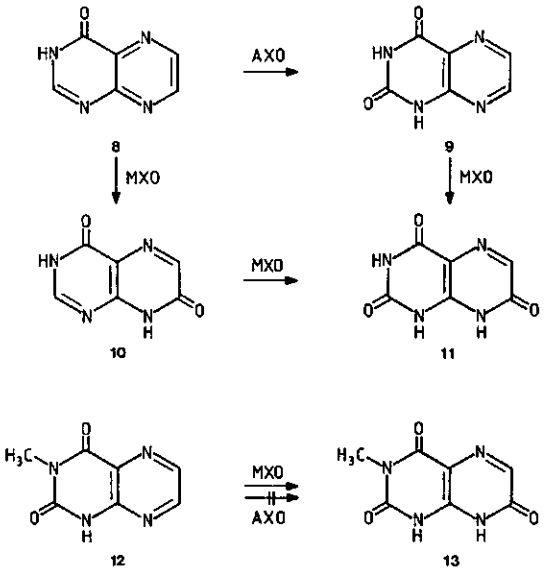
[a] Vm is expressed as percent of Vm of xanthine.
[b] NR = No Reaction.
[c] ND = Not Determined.

The Michaelis constant Km is strongly dependent on the pH: when reacted with MXO at pH=7.0 the Km of xanthine is 0.8 μM, at pH=8.0 Km= 5.3 μM, and at pH=8.5 a value of 28 μM is calculated [13].

7.3 4(3*H*)-PTERIDINONES

The difference in oxidation behaviour between AXO and MXO are even greater for 4(3*H*)-pteridinones than for purine derivatives. AXO oxidizes 4(3*H*)-pteridinone (**8**) regiospecifically at C-2 leading to the exclusive formation of lumazine (2,4(1*H*,3*H*)-pteridinedione (**9**)); in contrast oxidation of **8** with MXO [14] first lead to introduction of the hydroxygroup at C-7 *i.e.* **10** and then slowly at C-2 resulting in the formation of 2,4,7(1*H*,3*H*,8*H*)-pteridinetrione (**11**). The last mentioned compound could also be obtained by hydroxylation of **9** with MXO

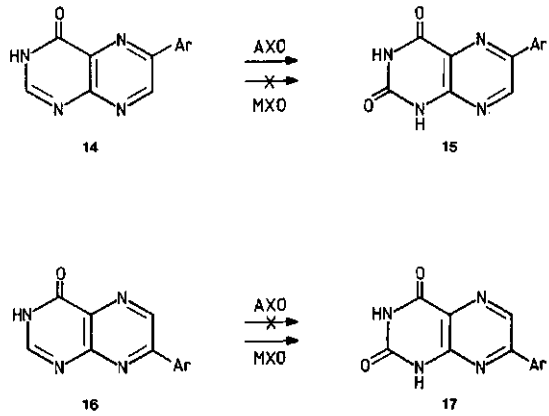
[14]. These data justify the conclusion that AXO in contrast to MXO is not able to oxidize the pteridine skeleton at position C-6 and C-7. This conclusion is in agreement with the experimental fact that 3-methyl-2,4(1*H*,3*H*)-pteridinedione (**12**), described to be oxidized [10] rather rapidly by MXO at C-7 giving **13**, is not oxidized by AXO. These reactions are summarized in Scheme 7.2.



Scheme 7.2

The difference in oxidation pattern of AXO and MXO is also very convincingly shown by the results of a study of the formation of aryllumazines by both enzymes. It was found that oxidation of 6-aryl-4(3*H*)-pteridinones (**14**) with the cell free extract [15] (prepared by ultrasonification and centrifugation of washed *Arthrobacter* cells adapted to xanthine) or with immobilized whole cells [16] gave a regiospecific hydroxylation at C-2 giving rise to 6-aryllumazines (**15**). This result is in sharp contrast to the behaviour of **14** towards MXO, where only a very slow oxidation has been observed.

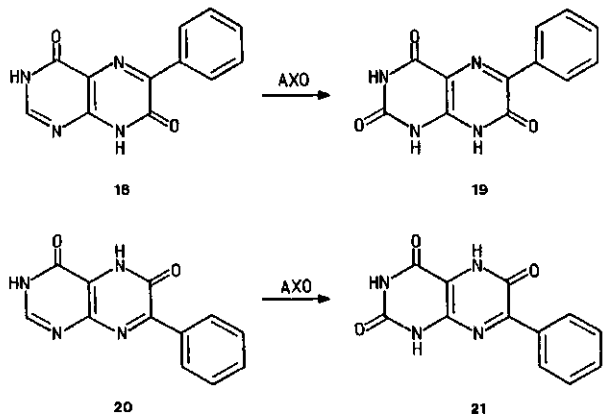
Interestingly the isomeric 7-aryl-4(3H)-pteridinones (**16**) are good substrates for MXO [17], but with exception of 7-phenyl-4(3*H*)-pteridinone (**16**, Ar = C_6H_5) and 7-(*p*-fluorphenyl)-4(3*H*)-pteridinone (**16**, Ar = *p*-FC_6H_4) no 7-aryl-4(3*H*)-pteridinones are oxidized by AXO.



14 15 16 17

Scheme 7.3

With AXO the oxidation rate for 6-phenyl-4(3*H*)-pteridinone is found to be that of 7-phenyl-4(3*H*)-pteridinone. It has been established in chapter 2 that AXO exclusively oxidizes C-2 in the 4(3*H*)-pteridinone system, regardless of substitution. Neither AXO nor MXO are able to oxidize aryl-4(3*H*)-pteridinone at the adjacent carbon of the pyrazine site. We also found that 4,7(3*H*,8*H*)-6-phenylpteridinedione (**18**) and 4,6(3*H*,5*H*)-7-phenylpteridinedione (**20**), when incubated with AXO (pH = 7.2, 100 μM), were oxidized at C-2 into the corresponding pteridinetriones **19** and **21** respectively. Comparing their oxidation rate with that of **14**, **18** was found to be oxidized at the same rate as **14** whereas **20** was converted at about 3% of the rate of **14** (Scheme 7.4).



18 19 20 21

Scheme 7.4

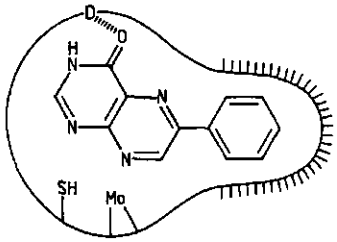
The resistance to oxidation of **14** by MXO and of **16** to AXO is not due to the absence of binding of these compounds in the active site of MXO or AXO. Recently it has been established that 6-aryl-4(3*H*)-pteridinones are very effective inhibitors [18] in the MXO-mediated oxidation of xanthine to uric acid and that 7-aryl-4(3*H*)-pteridinones can act as effective inhibitors of the oxidation of 1-methylxanthine into 1-methyluric acid catalyzed by the bacterial xanthine oxidase from *Arthrobacter* M-4.

All the results mentioned above convincingly show the important differences in substrate specificity and inhibition property between the enzymes MXO and AXO. As can be expected the enzyme-substrate affinity value Km for the oxidation of aryl-4(3*H*)pteridinones with both enzymes is found to be quite different. The Km constant for the 7-aryl-4(3*H*)-pteridinones with MXO (as deduced from Lineweaver-Burk plots) varies, depending on the substituent in the para position of the phenylring, between 0.5 and 1.8 μM (at pH=8.0) [17]. In comparison, the Km constant for the 6-aryl-4(3*H*)-pteridinones with AXO lies in the range of 7 to 14 μM (pH=8.0). From these data the cautious conclusion can be drawn that the affinity for the bacterial enzyme is certainly lower although one has to take into consideration that the substrates are different.

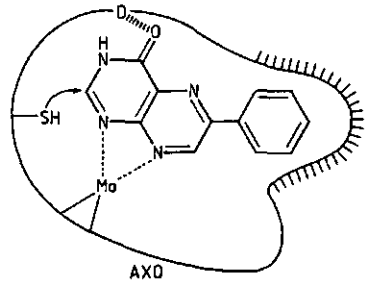
The Km-values of the reaction of the 6-aryl-4(3*H*)-pteridinones with AXO are about eight to twelve fold lower than the one found in the AXO-mediated oxidation of 4(3*H*)-pteridinone (108 μM). In comparison the Km-values for the 7-aryl-4(3*H*)-pteridinones with MXO [17] is about three to ten times lower than for 4(3*H*)-pteridinone at pH = 8.0 (5.6 μM) [10]. All these results present reasonable evidence for the existence of a hydrophobic site [19] in the vicinity of the active centre of both enzymes, and show that the hydrophobic effect in MXO seems to be somewhat stronger.

On consideration of the mechanism of the enzymatic oxidation there is ample evidence that the initial step in the hydroxylation involves a nucleophilic attack of a sulfur containing group, present in the active site close to the respective position of oxidation [20]. We suggest that the lack of reactivity of the 6-aryl-4(3*H*)-pteridinones towards MXO and of the 7-aryl-4(3*H*)-pteridinones towards AXO is due to a wrong position or orientation of the sulfur containing group because the hydrophobic site largely determines the affinity towards the enzyme (Scheme 7.5).

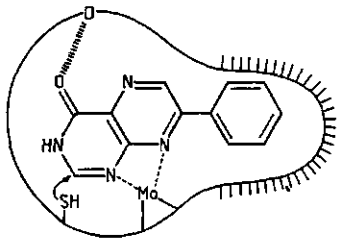
In our studies on the AXO-mediated oxidation we also included as substrates the 6-aryl-4(3*H*)-pteridinone-8-oxides (**22**). It was found that both the rate of oxidation of compounds **22** into the corresponding lumazines **23** and the affinity as expressed by Km are much lower than for 6-aryl-4(3*H*)-pteridinones.



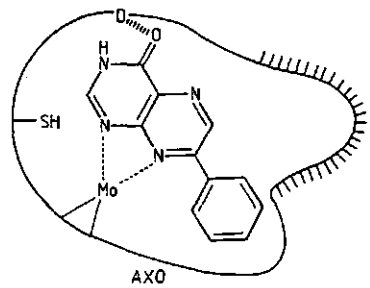
MXO
6-aryl
SH in wrong position



AXO
6-aryl
SH in right position



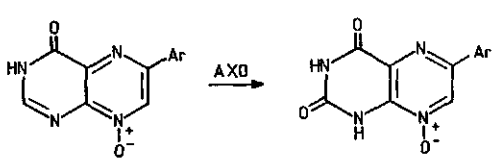
MXO
7-aryl
SH in right position



AXO
7-aryl
SH in wrong position

Scheme 7.5

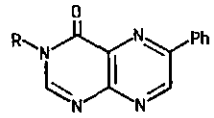
Although the kinetic parameters of these pteridine-8-oxides **22** are still under under study it is already evident that the Km constants vary between 30 and 50 μM and Vm is about 10 to 20% of that of **14**. Apparently due to the presence of an N-oxide function at N-8, the affinity to the bacterial enzyme is dramatically decreased. It is not possible to state, based on the experiments obtained so far, which of the following factor(s) is (are) mainly responsible for these results: (i) the decrease of the hydrophobic interaction in the active site of the enzyme, due to the polarity of the N-oxide group, (ii) the enhancement of the electron density at C-2, due to the electron donating character of the N-O moiety deactivating that position for nucleophilic attack.



22 23

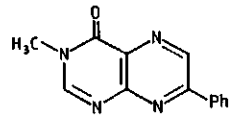
Scheme 7.6

As has been seen before in the purine series the affinity is decreased by the presence of methyl groups. It was also observed in the pteridine series that introduction of a methyl group at N-3 makes the compounds become refractory towards AXO. 3-Methyl-6-phenyl-4(3*H*)-pteridinone (**24**, R = CH_3), 3-methyl-7-phenyl-4(3*H*)-pteridinone (**25**) and 3-methyl-6-phenyl-4(3*H*)-pteridinone-8-oxide (**26**, R=CH_3) are slowly oxidized by the bacterial enzyme (with a rate of less than 1%). Increasing the size of the alkyl groups at N-3 by replacing methyl by ethyl, i-propyl, n-propyl and n-butyl in **24**, or by substituting methyl for ethyl, n-propyl, n-butyl and s-butyl in **26** respectively, results in complete deactivation. Apparently due to the increasing bulkiness of the alkyl group at N-3 there is no room for the substrate in the active site of the bacterial enzyme. In chapter 4 we also investigated the inhibitory capacity of compound **24** (R=CH_3) and **24** (R = C_2H_5) in the AXO-mediated oxidation of 1-methylxanthine to 1-methyluric acid. It is found that 3-methyl-6-phenyl-4(3*H*)-pteridinone is a non-competitive inhibitor and that the corresponding ethyl derivative is an uncompetitive inhibitor. Both compounds are better inhibitors than their 8-oxides.



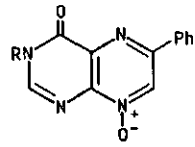
24

R= CH_3 , oxidation at C-2
R= C_2H_5 , n-C_3H_7, i-C_3H_7, n-C_4H_9
no oxidation by AXO



25

no oxidation by AXO at C-2



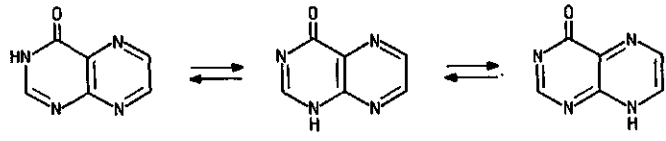
26

R= CH_3 , oxidation at C-2
R= C_2H_5 , n-C_3H_7 , n-C_4H_9 ,
s-C_4H_9
no oxidation

Scheme 7.7

It has been argued that the 4(1*H*)-pteridinone tautomeric form (**8b**) does not necessarily have to be the one which undergoes the oxidation in the active

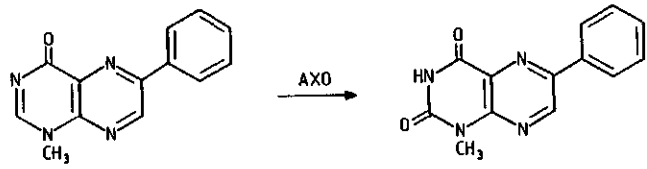
site of the bovine milk xanthine oxidase. The "active" form suggested is the tautomer 4(8*H*)-pteridinone (**8c**). The quinoid structure of the pyrimidine ring present in that tautomer is more vulnerable to oxidation [21]. This suggestion was born from the experimental fact that in reaction with MXO the 3-methyl-4(3*H*)-pteridinone [9] is attacked very slowly: the "active" form cannot be produced and therefore the rate of reaction is considerably reduced (Scheme 7.8).



8a 8b 8c

Scheme 7.8

Concerning the AXO-mediated oxidation of 3-methyl-6-phenyl-4(3*H*)-pteridinone **24** as discussed in chapter 4, our results do not contradict those proposals. In order to see whether the paraquinoid structure 4(1*H*)-pteridinone **8b** might also possibly be an "active form" for the bacterial enzyme we also investigated the oxidation of 1-methyl-6-phenyl-4(3*H*)-pteridinone **27** with AXO (pH = 7.2, 100 μM) and found that the oxidation rate of this compound is only 8% of that of 6-phenyl-4(3*H*)-pteridinone **14** but still faster than the 3-methyl derivative **24**. Thus, a contribution of the 4(1*H*) tautomer to the "active" form of 6-phenyl-4(3*H*)-pteridinone cannot be excluded.



27 28

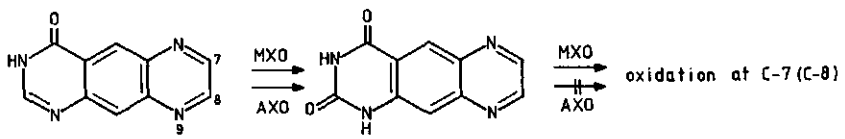
Scheme 7.9

7.4 *LINEAIR*-BENZO DERIVATIVES OF PTERIDINE

Lin-benzo derivatives of pteridine are compounds in which a benzene ring is inserted between the pyrimidine and pyrazine ring of pteridine *i.e.* **29**. In these systems terminal ring characteristics are preserved and due to the presence of the benzo-ring the potential for hydrophobic π-interaction is

increased. This increase in the lateral dimension should set limitations on the size and flexibility of enzyme binding sites specific for pteridine substrates.

Incubation of pyrazino[2,3-*g*]quinazoline-4(3*H*)-one (**29**) (*lin*-benzo-4(3*H*)-pteridinone) with MXO led to the formation of two products. The first product (**30**) is found to be converted further as indicated by the fact that the UV-spectra gave rise to other isosbestic points after only one minute. It suggests the formation of a trione. Only one product could be obtained with the cell free extract of *Arthrobacter* X-4. Since the UV-spectrum of that product could be superimposed upon that which was recorded after treatment of **30** with MXO for one minute, this indicates that *lin*-benzolumazine (**30**) is the first formed product. Only MXO is able to convert this substrate further into **31**, by attack at either the C-7 or C-8 position. This result again shows that the bacterial enzyme is more specific than MXO.



29 30 31

Scheme 7.10

7.5 CONCLUSIONS

Our observations and those of other investigators lead to the following conclusions:

(i) AXO differs from MXO in substrate specificity and shows a more specific oxidation pattern than MXO.

(ii) A hydrophobic region is present in both AXO and MXO; AXO usually binds the substrate less effectively than MXO.

(iii) AXO, unlike MXO, is only able to perform oxidation at the carbon position between both nitrogens in the pyrimidine ring. Therefore in the AXO-mediated reactions with 4(3*H*)-pteridinones no oxidation with AXO is observed in the pyrazine ring. The oxidation of hypoxanthine and xanthine to uric acid is an exception; this is understandable, they are natural substrates.

(iv) The results obtained with pteridines and its *lin*-benzo derivatives suggest that the geometry in the active site of MXO allows more freedom for substrate binding than AXO. The oxidation of **29** by MXO into **30** and **31** is illustrative as well as the exclusive conversion of **29** into **30** by AXO. After oxidation of **31** at C-2, a reorientation of the dione **31** in the active site of MXO can possibly take place, leading to binding in a different orientation and making oxidation in the pyrazine ring possible. The fact that AXO is not able to oxidize **30** suggests that a "good-fit" reorientation of dione **30** is not possible any more, indicating that the geometrical and spatial requirements in the active site of AXO are limited.

(v) A carbonyl group at C-4 is a structural requirement for efficient oxidation by AXO. This moiety most probably orientates the substrate molecule in the catalytic centre of the enzyme by coordination with an active site species (D). This interaction is of critical importance for a good approach of the enzymatic nucleophile in order to make oxidation possible. This requirement seems less critical for reactions with MXO since MXO is also able to oxidize the parent compounds purine and pteridine. It is evident that in the oxidation of these compounds one of the ring nitrogens can take over the role of the carbonyl moiety [5] in the oxidation of these compounds. Compared to **14** the bacterial xanthine oxidase from *Arthrobacter* M-4 purine and pteridine are oxidized at a rate of 3 and 1%, respectively. Obviously, the ring nitrogens cannot efficiently take over the role of the carbonyl moiety in AXO since it is stated in the literature that purine is not oxidized by bacterial xanthine oxidase from *Arthrobacter* S-2 [7-9].

Acknowledgements

We are indebted to mr. P.L.A.M. Corstjens for determination of the kinetic data and to dr. W. Kraus for his synthetic efforts.

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SUMMARY

In this thesis xanthine oxidase from *Arthrobacter* M-4 in the form of a cell-free extract or as immobilized cells has been studied with regard to its application in preparative organic chemistry. The enzyme has a broad substrate specificity towards azaheterocycles as purines and pteridines.

The unequivocal preparation of 6-aryl-4(3*H*)-pteridinones and 7-aryl-4(3*H*)-pteridinones with different substituents at the para position of the phenyl group is described. The oxidation of these compounds by (immobilized) xanthine oxidase from *Arthrobacter* M-4 usually goes fast for all the studied 6-aryl-4(3*H*)-pteridinones as well as for 7-(*p*X-phenyl)-4(3*H*)-pteridinones (X= H and F). All the compounds of the last mentioned series with a substituent larger than hydrogen or fluoro are slowly oxidized. Oxidation only takes place at C-2 of the pteridine nucleus. No oxidation at the pyrazine site is established. Small laboratory-scale oxidations have been carried out with cells entrapped in gelatine and crosslinked with glutaraldehyde. Based on spectral data the products of the oxidation reactions are 6- and 7-aryllumazines (chapter 2).

By comparison of the kinetic parameters of 6- and 7-phenyl-4(3*H*)-pteridinones with unsubstituted 4(3*H*)-pteridinone the existence of a hydrophobic pocket in the vicinity of the active site has been suggested. The rate-limiting step in the oxidation of 6-aryl-4(3*H*)-pteridinones by the bacterial xanthine oxidase is not significantly affected by the nature of the aryl substituent. The inhibition of the oxidation of 1-methylxanthine into 1-methyluric acid by 7-aryl-4(3*H*)-pteridinones is sensitive to electronic factors. A positive σ-value of 0.73 has been calculated for the inhibition indicating that a more electron-donating aryl substituent increases the affinity of this compound towards the enzyme (chapter 3).

Only 1-methyl-, 3-methyl-6-phenyl-4(3*H*)-pteridinone and 3-methyl-6-phenyl-4(3*H*)-pteridinone-8-oxide are found to be substrates althought their reactivity is still very low. The site of oxidation is not changed. 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 is a non-competitive inhibitor (Ki= 47 μM) whereas the 3-ethyl derivative is an uncompetitive one (Ki= 19.6 μM) (chapter 4).

The purified bacterial enzyme from *Arthrobacter* M-4 proves to be *monomeric*. This is quite remarkable since the flavoproteins to which xanthine oxidase belongs are usually dimeric. The inhibitory effects of 5,6-diaminouracil and bisalloxazine on the oxidation of xanthine into uric acid by bovine milk xanthine oxidase and xanthine oxidase from *Arthrobacter* M-4 have been examined. 5,6-Diaminouracil is about nine times more potent as inhibitor for xanthine oxidase from *Arthrobacter* M-4 than allopurinol. Inhibition constants are 8.4 μM for allopurinol and 0.98 μM for 5,6-diaminouracil. Bisalloxazine has a negligible inhibitory effect on the activity of both xanthine oxidases (chapter 5).

7-Phenyl-, 7-*p*-methoxyphenyl-, 7-methyl-, 7-t-butyl-, 6,7-diphenyl-, 6,7-dimethyl- and 2-phenylpteridine are converted in good yields into their respective 4-amino compounds when they are dissolved in liquid ammonia (-40°) and potassium permanganate has been added to the solution. Increase of the temperature of the amino-oxidation does not change the position of substitution, however the yields are lower. The intermediary of 4-aminodihydropteridines in these reactions has been proven by ^{1}H nmr spectroscopy (chapter 6).

By comparison of the substrate specificity between bovine milk xanthine oxidase and xanthine oxidase from *Arthrobacter* M-4 it is concluded that the hydrophobic site of the active site of both enzymes must be different. From the kinetic data it is clear that the bacterial enzyme has a less hydrophobic site (chapter 5) than the milk enzyme and that the bacterial enzyme only can perform oxidation at the pyrimidine site (chapter 7).

Despite the interesting complementary behaviour of the bacterial and the bovine milk enzyme towards both 6-and 7-aryl-4(3*H*)-pteridinones the only serious drawback for use of xanthine oxidase from *Arthrobacter* M-4 as an immobilized biocatalyst in heterocyclic chemistry is the low specific acitivity obtained after growing of these cells.

SAMENVATTING

In dit proefschrift wordt xanthine oxidase uit *Arthrobacter M-4* bestudeerd, zowel in de de vorm van celvrij extract als geimmobiliseerde cellen, met het oog op toepassing in preparative organische synthese. Dit enzym heeft een brede substraat specificiteit tegenover azaheterocyclische verbindingen als purines en pteridines.

De eenduidige synthese wordt beschreven van 6-aryl-4(3*H*)-pteridinonen en 7-aryl-4(3*H*)-pteridinonen met verschillende substituenten op de para positie. De oxidatie van deze verbindingen door (geimmobiliseerde) xanthine oxidase uit *Arthrobacter* M-4 verloopt gewoonlijk snel voor alle bestudeerde 6-aryl-4(3*H*)-pteridinonen zowel als voor 7-(*p*X-phenyl)-4(3*H*)-pteridinonen (X= H of F). Alle verbindingen van de laatst genoemde reeks met een substituent groter dan waterstof of fluor worden traag geoxideerd. Oxidatie vindt enkel op C-2 van het pteridine skelet plaats. In de pyrazine ring wordt geen oxidatie vastgesteld. Kleinschalige laboratorium oxidaties werden uitgevoerd met cellen ingebed in gelatine en gecrosslinkt met glutaaraldehyde. Gebaseerd op spectrale gegevens zijn de oxidatie produkten 6- en 7-aryllumazines (hoofdstuk 2).

Door vergelijking van de kinetische parameters van 6- en 7-fenyl-4(3*H*)-pteridinon met ongesubstitueerd 4(3*H*)-pteridinon wordt het bestaan van een hydrofoob gebied in de omgeving van het actieve centrum gesuggereerd. De snelheidsbepalende stap in de oxidatie van 6-aryl-4(3*H*)-pteridinones door het bacteriëel xanthine oxidase wordt niet noemenswaardig beinvloed door de aard van de aryl substituent. De remming van de oxidatie van 1-methylxanthine tot 1-methylurinezuur door 7-aryl-4(3*H*)-pteridinonen is gevoelig voor electronische factoren. Een positieve σ -waarde van 0.73 is gevonden voor de remming, wat aangeeft dat de aanwezigheid van een elektronenstuwende aryl substituent de affiniteit van deze verbinding voor het enzym verhoogt (hoofdstuk 3).

Enkel 1-methyl-, 3-methyl-6-fenyl-4(3*H*)-pteridinon en 3-methyl-6-fenyl-4(3*H*)-pteridinon-8-oxide zijn substraten alhoewel hun reactiviteit nogal laag is. De oxidatiepositie wordt niet gewijzigd. Alle 3-alkyl derivaten worden minder stevig gebonden aan het enzym dan 6-fenyl-4(3*H*)-pteridinon. De invoering van een N-oxide op N-8 verlaagt in hoge mate de binding van de substraten. Remming studies hebben aangetoond dat 3-methyl-6-fenyl-4(3*H*)-pteridinon een niet-competitieve remmer is (Ki= 47 μM) terwijl het 3-ethyl derivaat een oncom-

petitieve remmer is (Ki= 19.6 μM) (hoofdstuk 4).

Het gezuiverde bacteriële enzym van *Arthrobacter* M-4 blijkt een *monomeer* te zijn. Dit is erg merkwaardig daar flavine eiwitten waartoe xanthine oxidase behoort, gewoonlijk *dimeren* zijn. Het remmend effect van 5,6-diaminouracil en bisalloxazine op de oxidatie van xanthine tot urinezuur door xanthine oxidase uit koemelk en uit *Arthrobacter* M-4 werden onderzocht. 5,6-Diaminouracil is een ongeveer negen maal sterkere remmer dan allopurinol voor xanthine oxidase van *Arthrobacter* M-4. Remmingsconstanten zijn 8.4 μM voor allopurinol en 0.98 μM voor 5,6-diaminouracil. Bisalloxazine heeft een verwaarloosbaar remmend effect op de activiteit van beide enzymen (hoofdstuk 5).

7-Fenyl-, 7-*p*-methoxyfenyl-, 7-methyl-, 7-t-butyl-, 6,7-difenyl-, 6,7-dimethyl- en 2-fenylpteridine worden in hoge opbrengsten omgezet in hun respectievelijke 4-amino verbindingen wanneer deze zijn opgelost in vloeibare ammoniak (-40°) waaraan kaliumpermanganaat is toegevoegd. Verhoging van de temperatuur in het aminering-oxidatie proces verandert de amineringspositie niet, de opbrengsten zijn echter lager. De 4-aminodihydropteridines zijn intermediairen in deze reacties zoals is aangetoond door ^{1}H nmr spectroscopie.

Door vergelijking van de substraat specificiteit van runder melk xanthine oxidase en xanthine oxidase van *Arthrobacter* M-4 wordt besloten dat het hydrofoob gebied in het actieve centrum van beide enzymen verschillend is. Uit de kinetische gegevens is het duidelijk dat het bacteriële enzym een minder hydrofoob deel (hoofdstuk 5) heeft dan het melk enzym en dat het bacteriële enzym enkel oxidatie in de pyrimidine ring kan uitvoeren (hoofdstuk 7).

Ondanks het interessante complementair gedrag van zowel het bacteriële als het runder melk enzym tegenover 6- en 7-aryl-4(3*H*)-pteridinonen, is het enige ernstige nadeel voor gebruik van xanthine oxidase uit *Arthrobacter* M-4 als geïmmobiliseerde biocatalysator in heterocyclische chemie, de lage specifieke activiteit verkregen na het kweken van deze cellen.

CURRICULUM VITAE

De auteur van dit proefschrift werd op 25 oktober 1956 geboren te Deinze (België). Middelbaar onderwijs werd genoten aan het Sint-Thomascollege te Antwerpen Linkeroever waar het diploma richting Wetenschappelijke A werd behaald in juni 1974. In oktober 1974 nam mijn studie aan de faculteit van de Landbouwwetenschappen aan de Rijksuniversiteit Gent een aanvang. Het kandidaatsdiploma van landbouwkundig ingenieur werd behaald in juni 1976. Als keuzepakket tot het behalen van de graad van Ingenieur voor de Scheikunde en Landbouwindustrieën werd een grondige studie van de Theoretische en Fysische scheikunde gekozen. Het werk van einde studiën, getiteld "Vergelijking tussen de begassing met ethyleenoxide en gamma-irradiatie van enkele specerijen" werd begeleid door prof.ir. L. Baert en prof.dr.ir. A. Huyghebaert. Deze studie werd afgerond in september 1979. In de periode van januari 1980 tot december 1986 werd het in dit proefschrift beschreven onderzoek uitgevoerd onder leiding van prof.dr. H.C. van der Plas. In diezelfde periode was ik als tijdelijk wetenschappelijk medewerker verbonden aan de vakgroep Organische Chemie van de Landbouwuniversiteit Wageningen waarbij naast begeleiding van aan onderzoek werkende stagiair(e)s, ook onderwijs aan kandidaats- en ingenieursstudenten werd gegeven.