STUDIES OF THE QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIP OF THE INHIBITION OF XANTHINE OXIDASE BY AZAHETEROCYCLIC COMPOUNDS

ter nagedachtenis aan mijn
vader en schoonmoeder

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doctor in de landbouw- en milieuwetenschappen,
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dr. H. C. van der Plas,
in het openbaar te verdedigen
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STELLINGEN

1 Bushelev en Stepanov onderbouwen de QSAR vergelijking voor de antitumor werking van enkele anthracyclines slechts met de correlatie coëfficiënt r. Om de betrouwbaarheid van de QSAR vergelijking te onderbouwen zijn bovendien de F waarde, de t waarde, de standaard deviatie s en de intercorrelatie coëfficiënten gewenst.

S.N. Bushelev en N.F. Stepanov (1989). *Z. Naturforsch.*, **44C**, p. 212-216
Dit proefschrift, hoofdstuk 1.4.

2 Om de interacties van verschillende heterocyclische stoffen met xanthine oxidase te kunnen vergelijken moet men eerst zekerheid hebben over de manier waarop deze stoffen aan het actieve centrum van xanthine oxidase binden.

Dit proefschrift, hoofdstuk 6.2.

3 Het bijprodukt van de reactie van 3ß,17ß-diacetoxy-6ß-chloro-5-methyl-19-nor-5ß-androst-9-ene met lithium aluminium hydride is waarschijnlijk niet 4a-methyl-A-homo-B,19-dinor-5ß-androst-9-ene-3ß,17ß-diol, zoals Kasal beweert, maar de gedehalogeneerde uitgangsstof.

A. Kasal (1989). *Collect. Czech. Chem. Commun.*, **54**, p. 1318-1326.
J.B.P.A. Wijnberg, L.H.D. Jenniskens, G.A.Brunekreef, Æ. de Groot (1990). *J. Org. Chem.*, **55**, p. 941-948.

4 De conclusie van Patel en Fernandes, dat enkele door hen gesynthetiseerde 5-arylazo-4-thiazolylacetaten en 5-aryl-1,5-dihydro-6-nitropyrazolo[4,3-c]pyrazoles een antibacteriële werking hebben, wordt onvoldoende aangetoond met experimentele gegevens.

H.V. Patel en P.S. Fernandes (1989). *Indian J. Chem.*, **28B**, p. 56-60.
H.V. Patel en P.S. Fernandes (1989). *J.Indian Chem. Soc.*, **66**, p. 327-329.

5 De kanttekening van Lewis e.a. bij de depolymerisatie van lignine door mierikswortel peroxidase in een organisch medium had Dordick in zijn overzichtsartikel behoren te bespreken.

J.S. Dordick (1989). *Enzyme Microb. Technol.*, **11**, p. 194-211.
N.G. Lewis, R.A. Razab en E. Yamamoto (1987). *Proc. Natl. Acad. Sci.*, **84**, p. 7925-7927.
J.S. Dordick, M.A. Marletta, A.M. Klibanov (1986). *Proc. Natl. Acad.. Sci.*, **83**, p. 6255-6257.

6 Het is onwaarschijnlijk, dat bij de nitrering van 2-amino-5-fenylpyrimidine alleen maar 2-amino-5-(*p*-nitrofenyl)pyrimidine ontstaat.

D.J. Brown en B.T. England (1971). *J. Chem. Soc. (C)*, p.425-431.

7 De verklaring, die Maruyama en Kawabata geven voor de verschuiving van de Soret band in de ortho gesubstitueerde polyyne porfyrines bij een toename van het aantal geconjugeerde acetyleen-eenheden, is uitermate speculatief.

K. Maruyama en S. Kawabata (1990). *Bull. Chem. Soc. Jpn.*, **63**, p.170-175.

8 De grote hoeveelheid softwarepakketten, die beschikbaar zijn om chemische structuren te tekenen, molecuul modellen te maken en interacties tussen chemische stoffen te bestuderen op een beeldscherm leiden langzamerhand tot "door de bomen het bos niet meer kunnen zien".

N.C. Cohen, J.M. Blaney, C. Humblet, P. Gund en D.C. Barry (1990). *J. Med. Chem.*, **33**, p. 883-894.
D.E. Meyer (1988). in W.A. Warr (edit.) *Chemical Structures, the International Language of Chemistry*, Springer Verlag, Berlijn, p. 251-259.

9 De overtreders van het rookverbod in openbare ruimten van rijksgebouwen behoren waarschijnlijk tot dezelfde groep hardleerse en weinig met de omgeving rekening houdende mensen als de overtreders van het verbod om harder te rijden dan 120 km per uur op de rijkswegen.

Han Naeff

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Studies of the quantitative structure-activity relationship of the inhibition of xanthine oxidase by azaheterocyclic compounds

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1. INTRODUCTION

1.1 GENERAL

The use of enzymes in organic chemistry has become accepted practice during the last fifteen years, and many laboratories are now using them to introduce functional groups into organic molecules. Enzymes have the unique ability to catalyze reactions that would otherwise be difficult (if not impossible) to perform with conventional chemical methods. Moreover, these reactions have a high rate, a high specificity, and can be performed under mild conditions.

Enzymatic capabilities have been a major topic of research in this University's Department of Organic Chemistry since 1975. Experiments have, for example, used enzymes in regio-specific conversions of azaheterocyclic compounds. These reactions involved studying the biocatalysts bovine milk xanthine oxidase [1], bacterial xanthine oxidase [2], chicken liver xanthine dehydrogenase [3], rabbit liver aldehyde oxidase [4], and haloperoxidases [5]. Experiments with the xanthine oxidase used the azaheterocycles 6-aryl-substituted pteridin-4-one, 7-aryl-substituted pteridin-4-one, 7-aryl-substituted linear-benzo derivative of pteridin-4-one, and 1-aryl-substituted nicotinamide [6].

In the Department's work, if enzymes are used as catalysts, the organic molecules act as enzyme substrates. In other types of research, the organic molecules act as inhibitors of an enzymatic reaction. Inhibitors have become important research tools in biochemistry, biophysics, medicine, and pharmaceutics. They are used to study specific intracellular functions and elucidate the mechanism of ligand binding, the process of enzymatic catalysis, and the structure of the enzymatic active site. A favored goal of anti-parasite and anti-cancer chemotherapy is the inhibition of an enzyme essential to bacterial growth or tumor-like cell division. Partial or total inhibition of a mammalian enzyme to control the functioning of specialized cells (e.g. of the brain and nervous system) is a specific goal of medicinal chemistry.

The first step in designing therapeutic or pharmaceutical compounds is to identify a target enzyme that, when inhibited, will produce the desired effect without toxic side effects. After the target enzyme has been identified, a strategy to find and synthesize an effective inhibitor must be planned.

Even though many of the enzyme inhibitors now widely used as medicines were found by trial and error, it is estimated that this method produces only one useful drug for every three to five thousand compounds synthesized [7]. Recent discoveries, however, have provided a more rational basis for designing enzyme inhibitors.

Biological activity is no longer considered solely a function of chemical structure. Nowadays, it is a recognized function of physicochemical properties. This has led the way to the development of a relationship between the structure and biological activity of a compound. The ultimate goal in the study of that relationship is the realization of a "tailor-made" bioactive molecule.

1.2 A SHORT HISTORY OF QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIP (QSAR) STUDIES

Researchers have been using physicochemical properties to study the biological activity of organic compounds since the 1880's. Even at that early date, the researchers realized that, because of its reliance on structural formulas, the language of synthetic organic chemistry was not the most suitable one for understanding structure-activity relationships (SAR's) [8]. Accordingly, those first studies, which compared only one molecular property with the biological activity, were often unsuccessful.

Over the last thirty years, it has become clear that the biological activity of a compound may depend on more than one physicochemical property (or other process) of the biological object. These include permeation and transport, drug metabolism, and interaction with the biological organism. This thesis limits discussion of the SAR to the physical and physicochemical properties of an organic compound; permeation and transport, and drug metabolism in the SAR fall outside of its scope.

An SAR study has two purposes, namely prediction and diagnosis. Prediction, as the word implies, is the extrapolation and interpolation of a correlation study. In a correlation study, one tries to optimize the bioactivity of a compound by varying the substitution pattern in a congeneric group of compounds. Research in physical organic chemistry has added significantly to the understanding of substituent properties. Diagnosis is an attempt to answer questions about the interaction between drugs and receptors. New

tools (e.g. X-ray analysis, high-resolution nuclear magnetic-resonance spectroscopy, electron spin-resonance spectroscopy, and computer-aided molecular graphics) have greatly facilitated the study of the interaction between small organic compounds and biological macromolecules like enzymes and DNA [9]. The ultimate objective of diagnosis is the rational development and designing of entirely new classes of compounds for specific biological activities.

The SAR study came into its own in the 1960's, as computers were becoming widely available. For the first time, it was possible to use more than one parameter in an SAR study and calculate a quantitative relationship between a biological activity and the parameters describing a congeneric group of compounds. Since then, numerous methods for studying quantitative structure-activity relationships (QSAR's) have been developed and commonly applied. These include the nonparametric methods of Free and Wilson [10] and Fujita and Ban [11], the parametric method of Hansch and co-workers [12], discriminant analysis [13], and pattern recognition [14].

The choice of a method depends on several factors (e.g. the quality of biological data, the number of compounds tested, the degree of variance in the results, and the ratio of the time required for synthesis and biological testing). The most widely used method is the linear free-energy approach, also known as the Hansch approach [12]. It attributes variation in a biological activity (BA) to the different substituents whose different physicochemical properties modify a BA relative to the parent compound. These properties can be translated into parameters and then added and combined. The parameters are adopted from physical chemistry and are assumed to be electronic, steric, hydrophobic, and dispersive (see Table 1.1). A more detailed discussion follows in the next section.

The most widely used substituent parameters are the Hammett constant (σ), the Taft steric constant (E_S), the hydrophobic constant (π), and the molar refractivity (MR). Although Hansch and Leo [15] have tabulated the values of many substitution constants, the data base is far from complete. The Hansch approach results in the following equation:

$$BA = a + b\pi + c\sigma + dE_S + eMR \qquad (1.1)$$

Table 1.1 Linear Free-Energy-Related Parameters

Parameter	Name	Description	Ref.
HYDROPHOBIC PROPERTIES			
P	Partition coefficient	Log P, taken as a measure of the hydrophobicity of the molecule; to measure P, it is best to use an octanol-water system.	16,17
π	Hydrophobic constant	$\pi = \log P_X - \log P_H$, where P_X is the partition coefficient of the substituted compound, and P_H the partition coefficient of the unsubstituted reference compound.	17
R_M	Hydrophobic constant from chromatography	Log P is linearly related to R_M, as expressed by $\log P = R_M + a$.	18
k'	Hydrophobic constant from HPLC	Log P is linearly related to log k' (for reversed-phase systems), as expressed by $\log P = k' + a$.	19
ELECTRONIC PROPERTIES			
$\sigma(\sigma_m, \sigma_p)$	Hammett constant	Defined only for **meta** and **para** substituents in aromatic rings to represent electronic character; a positive value for σ denotes an electron-withdrawing character, a negative value for σ denotes an electron-donating character.	5
σ^*	Taft constant	Measure of the electronic effect produced by aliphatic substituents.	21
$\mathcal{R}$ $\mathcal{F}$	Resonance and Field constants	σ involves the resonance effect ($\mathcal{R}$) and field (inductive) effect ($\mathcal{F}$) of a substituent. In other words, $\sigma = a\mathcal{R} + b\mathcal{F}$, where a and b are constants, depending upon the type of σ (σ_m, σ_p); $\mathcal{R}$ and $\mathcal{F}$ are more indicative of the intrinsic resonance and field effects of a substituent; the sign of $\mathcal{R}$ and $\mathcal{F}$ indicates the sign of the charge that the substituent would place on the ring.	22
pK_a	Ionization constant	$pK_a = -\log K_a$, where K_a is the ionization constant of an acid.	
DISPERSION PROPERTIES			
MR	Molar refractivity	$MR = [(\eta^2-1)/(\eta^2+2)]MW/\rho$, where η is the refractive index for the sodium D line, MW is the molecular weight, and ρ is the density of the compound; MR can be used as a steric parameter in the absence of E_S; MR also measures the electronic effect and can reflect the dipole-dipole interaction at the active site.	23,24
STERIC PROPERTIES			
E_S	Taft steric constant	Related to the acid-catalyzed hydrolysis of α-substituted acetates (XCH_2COOR); it represents the steric effect influencing intramolecular and intermolecular hindrance to the reaction or binding.	21
MV	Molar volume	General steric bulk parameter.	25
V_w	v.d.Waals volume	Steric bulk parameter based on Bondi radii.	26
r	v.d.Waals radius		27
R	Interatomic distance		28
L, B_1, B_5	Sterimol parameters	Directional steric parameters, where L is a measure of the length of the substituent, B_1 a measure of its smallest width, and B_5 a measure of its largest width.	29
χ	Molecular connectivity index	Topological parameter defined to account for the effects of atoms, bonding types, adjacent spaces, branching patterns, unsaturation, and heteroatomic content in a molecule on the reactivity or activity of that molecule.	30
MTD	Minimum topological difference	Deviation from an optimum standard molecule.	31

where BA is the concentration (C), that is required to produce some standard biological response. BA is often expressed as the inverse of C, or as the logarithm of the inverse of C. The values of the coefficients a, b, c, d, and e in Equation 1.1 are fit by least-squares multiple-regression analysis. This method also provides a measure of:

- The **t** value, which gives an indication of the significance of the coefficient of each parameter;
- The value of the $\mathbf{r^2}$ statistic, which is that fraction of the variance in the BA data that is explained by the equation;
- The **s** value, which is the standard deviation of the observed BA values;
- The **F** value, which is the ratio between the variances of the observed and the calculated BA values, and which indicates the significance of the total equation.

After performing the multiple regression analysis, one examines the data set for interesting equations (i.e. those that contain statistically significant terms, make mechanistic sense, and do not overfit the data). This last quality means that, in general, only one parameter is used for a minimum of three observed BA values.

1.3 PARAMETERS USED IN QSAR ANALYSIS

1.3.1 Hydrophobic Parameters

Interest focused on the hydrophobic property of a compound when Meyer [32] and Overton [33] showed that the narcotic activity of many simple organic compounds paralleled their oil-water partition coefficient (P). In QSAR analysis, the logarithm of the partition coefficient (log P) is commonly used as a hydrophobic parameter. Octanol-water solutes are now the standard reference system for measuring P [17].

Although log P is a measure of the hydrophobicity of a whole molecule, it is not unusual to work with a congeneric group of compounds, in which a large portion of the structure remains constant. In that case, one need not know the hydrophobicity of the entire compound; knowing only the relative hydrophobicity of the substituents can be enough to perform the QSAR analysis. To find the relative hydrophobicity of substituents, Leo et al. [17]

and Fujita et al. [34] defined the hydrophobic constant (π) analogous to the Hammett constant (σ), in which:

$$\pi_X = \log P_X - \log P_H \qquad (1.2)$$

where P_X is the partition coefficient of the substituted compound and P_H is the partition coefficient of the parent compound. A positive value for π means that, relative to H, the substituent favors the octanol phase. A negative value indicates the substituent's hydrophilic character relative to H.

1.3.2 Electronic Parameters

Physical organic chemists are studying many electronic parameters that are based on linear free-energy relationships. In QSAR analysis, an important parameter is the Hammett constant [15], which is defined as:

$$\sigma = \log K_X - \log K_H \qquad (1.3)$$

where K_H is the ionization constant for benzoic acid in water at 25°C and K_X is the ionization constant for both **meta** and **para** derivatives under the same experimental conditions. If σ is positive, the substituent withdraws electrons from the aromatic ring. If it is negative, electrons are released to the ring. Presumably, the electronic effect has two components: a through-bond electrostatic effect of the substituent on a reaction center (inductive effect) and a direct through-space electrostatic effect of the substituent on a reaction center (field effect). Taft [21] has defined the inductive constant σ^*, which is valid for aliphatic systems, and Swain and Lupton [22] have defined the resonance constant ($\mathcal{R}$) and the new field constant ($\mathcal{F}$).

1.3.3 Dispersion Parameters

Nonspecific interactions (e.g. the hydrophobic effect and dispersion forces) can be very important in biological activities. It is likely that nonspecific interactions are crucial in the early stages of substrate and

receptor interaction, when the more specific stereoelectronic factors have not yet come into play.

In their classic study of the interaction of small organic compounds with macromolecules, Pauling and Pressman [35] reported that dispersion forces are related to molar refractivity (MR). This relationship is expressed as:

$$E = \frac{3\,\alpha_a\,\alpha_b}{2\,r^6} * \frac{I_a I_b}{I_a + I_b} \qquad (1.4a)$$

$$MR = \frac{4\pi N\alpha}{3} \qquad (1.4b)$$

where E is the cohesive energy between two atoms (**a** and **b**), α is the polarizability of those atoms, r is the distance between **a** and **b**, and I is the ionization potential. Equation 1.4b shows the relationship between MR and α and, by extension, that between MR and E. In experiments, MR is obtained with the Lorentz-Lorentz equation:

$$MR = \frac{\eta^2 - 1}{\eta^2 + 2} * \frac{MW}{\rho} \qquad (1.4c)$$

where η is the refractivity index, ρ is the density, and MW is the molecular weight of a compound. Its relationship to MW makes MR a steric bulk parameter. Because MR is an additive-constitutive molecular property, it is possible to calculate fragment values for many common groups of atoms. Hansch et al. [23] have compiled an extensive listing of MR values.

MR has not always been practical for QSAR analysis. If the set of congeners to be studied is not designed carefully, π and MR often turn out to be so highly collinear that they yield approximately the same correlation.

1.3.4 Steric Parameters

Researchers have been using the steric effects of substituents to study organic reaction rates since the latter half of the nineteenth century [36]. The first generally accepted numerical definition of steric effects was

formulated by Taft [21]. It reads:

$$E_S = \log (k_X / k_H)_A \qquad (1.5a)$$

where E_S is the steric constant and k refers to the rate constant for the acid hydrolysis (A) of type **I** esters:

$X - CH_2COOR$

I

The acid hydrolysis method cannot be used with some substituents because the esters are not stable under experimental conditions. For those substituents, Kutter and Hansch [27] calculated E_S with the following correlation equation:

$$E_S = -1.839\ r_v(av) + 3.484 \qquad (1.5b)$$

where $r_v(av)$ is the average of the maximum and minimum van der Waals radii of the substituent, as estimated according to Charton [28].

Other descriptors of steric effects are the van der Waals volume (V_w) [26], the van der Waals radii [27], and the Molar volume [25a], all of which are based on the atomic radii calculated by Bondi [25b]. Also in this group is the molecular connectivity index (χ) [30], which gives a quantitative description of molecule branching. Comparison of χ with various steric parameters has, in some cases, revealed a strong intercorrelation [37], suggesting that χ is also a measure of the substituent steric bulk.

Despite the many descriptors, it is very difficult to obtain a complete and accurate description of the steric interaction between a complex substituent and a macromolecular surface. The descriptors named above generally apply very well to spherically symmetric substituents. To obtain a description of asymmetric substituents, Verloop, Hoogenstraaten, and Tipker [29] developed a multiparameter method called Sterimol (Figure 1.1)

Sterimol uses five parameters: B_1, B_2, B_3, B_4, and L. The first four parameters are the widths of a substituent measured in four rectangular directions. The fifth parameter, L, is the length of the substituent along the

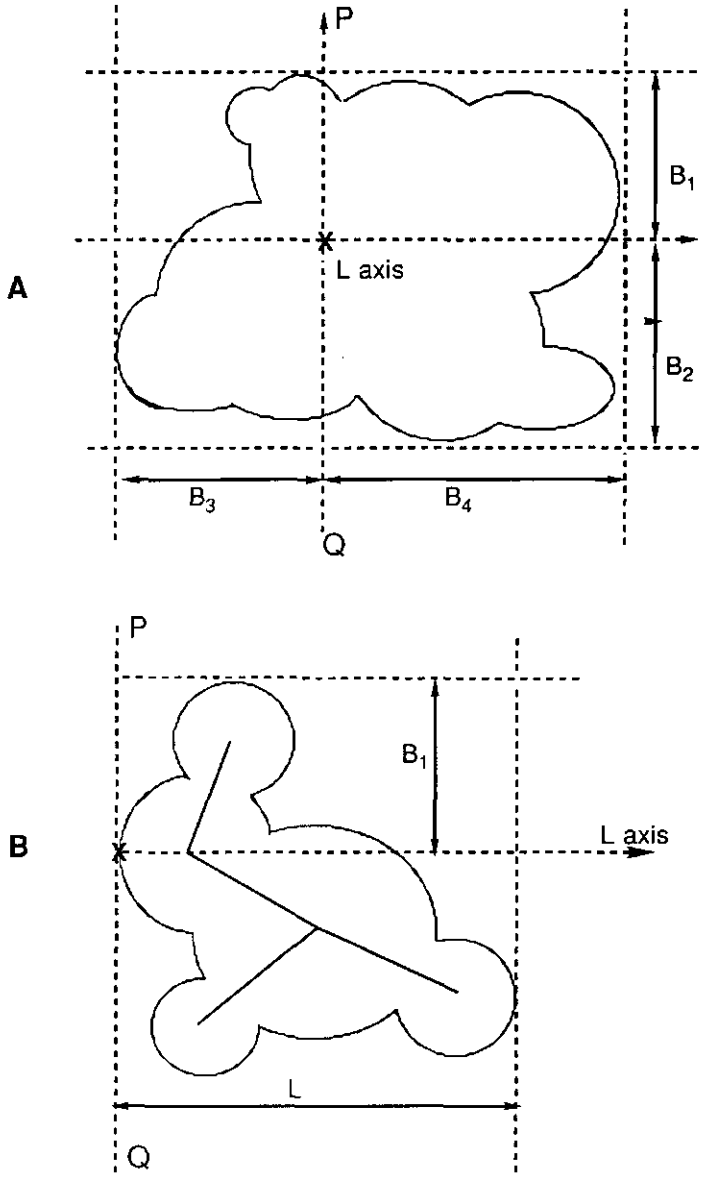


Figure 1.1 Selection of Sterimol parameters for a substituent.

axis of the bond between the first atom of the substituent and the parent molecule. For calculating the steric dimensions, Sterimol includes a computer program that uses van der Waals radii, standard bond lengths and angles, and logical conformations.

A detailed QSAR study requires five Sterimol parameters to account for steric effects. A statistically justified investigation requires fifteen congeners to analyze steric factors. A biological activity, however, rarely depends on steric factors only, so electronic, hydrophobic, and dispersion factors must also be considered. More congeners are required to obtain a meaningful QSAR, which is especially true when two or more substituent positions are present in the congeneric compound group. To accommodate the extra congeners, Verloop [38] reduced the number of parameters to three when updating Sterimol. These are L, B_1, and B_5, with L still representing the length of the substituent, B_1 the smallest width, and B_5 the largest width. B_5 is often equal to parameter B_4 of the original Sterimol [24].

1.3.5 Minimum Topological Difference (MTD) Calculations

Another parameter for describing steric substituent effects is the minimum topological difference (MTD). Simon et al. [39] defined the minimum steric difference (MSD) between a compound and the natural substrate of a biological system as the non-overlapping volume of the lowest energy conformation of the two molecules. In practice, this means that the planar structural formulas of the molecules are superimposed and that the non-superimposable atoms are counted (Figure 1.2). One method of estimating the deviation from the ideal bulk as represented by the natural substrate is to characterize the MSD parameters. There are, however, certain problems inherent in this method. These are:

- The subjectivity in superimposing the molecules;
- The difficulty of defining the natural substrate, which is often unknown in QSAR studies. Simon et al. adopted the most active molecule of a congeneric group as the standard, assuming that its shape would be most like that of the "natural" substrate;
- The lack of discrimination between differences in the shape of the molecule directed towards the binding site of the receptor (which would be relevant) and differences in the shape towards other directions (which would be irrelevant).

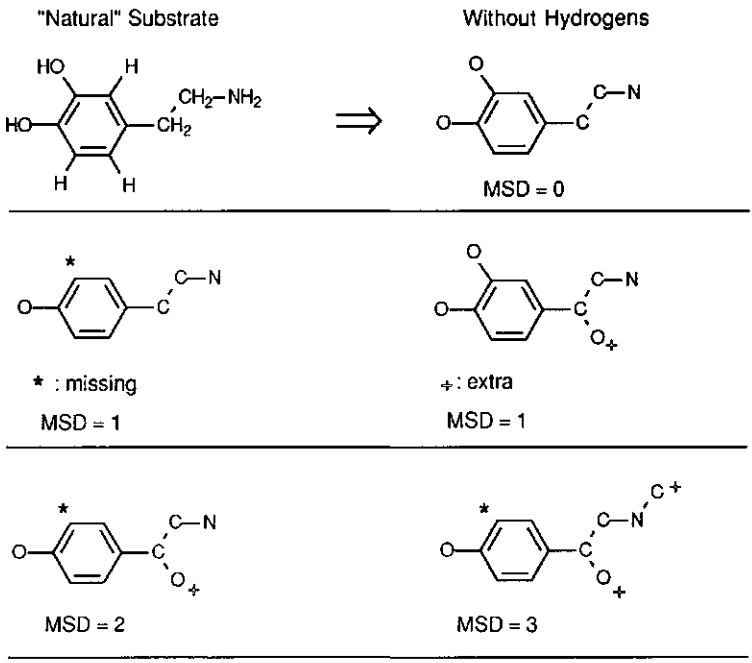


Figure 1.2 Calculation of the MSD parameters (Reference 40).

Recently, Balaban et al. [31] described a method that overcomes these problems to a certain extent. Called the minimum topological difference (MTD) method, it is based on the development of an optimal standard molecule by systematic analysis of the shapes of the members of a congeneric group in relation to their biological activities. The method consists of the following steps:

- Develop a so-called "hypermolecule" (i.e. a molecule that is the assemblage of all atomic positions of all molecules in the congeneric group). Figures 1.3 and 1.4 illustrate this for some substituted benzonitriles;
- Choose an initial standard molecule (e.g. the most active member) and calculate the MTD values of all the members using the MSD method. All the positions in the hypermolecule will now change, one by one, until they have been accounted for, favorable, unfavorable, and indifferent (i.e. not adding to the MTD value at all). After each change, correlate the MTD values with the biological activity and optimize the correlation

coefficient using the steepest ascent method. If no single change in the final standard can produce MTD values that correlate better with the biological activity, derive an optimum standard;

- Repeat the first two steps several times, starting from different initial standards (e.g. the entire hypermolecule or initial standards that have been randomly generated) until most optimizations have led to the same standard. This will avoid the results indicating only a local optimum of the standard.

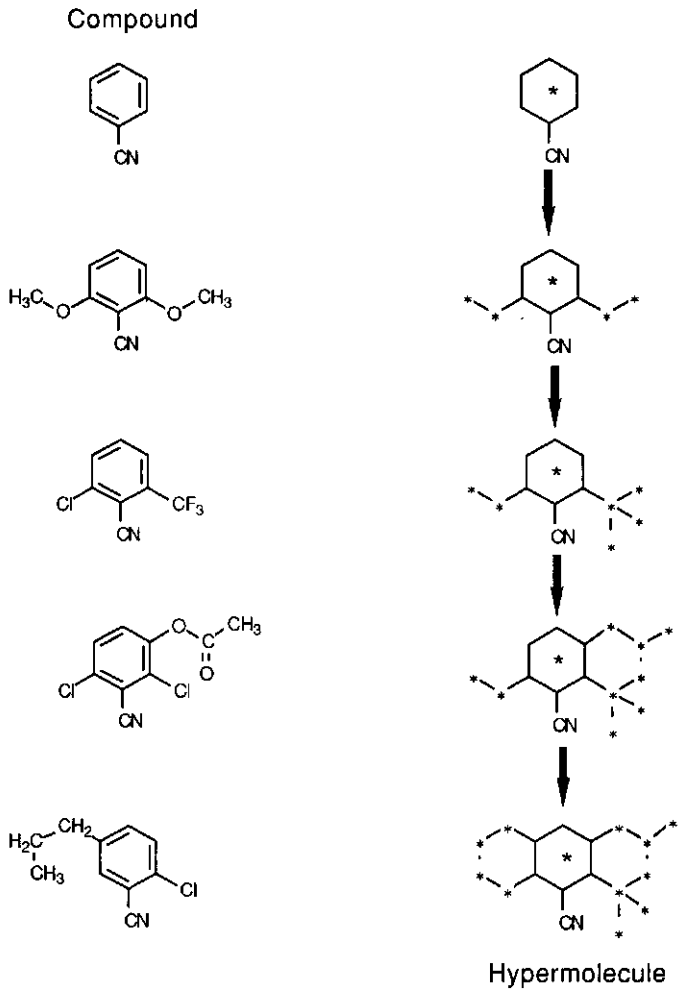
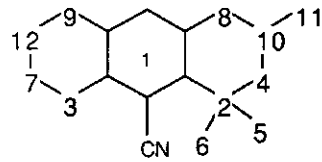


Figure 1.3 Construction of the hypermolecule of some substituted benzonitriles (Reference 40).

Balaban et al. accounted for the additional influence of electronic, hydrophobic, and dispersion effects by adding σ, π, and MR to the regression after developing an optimum standard. In contrast, Tipker and Verloop [40] preferred to add those parameters sooner, while developing the optimum standard. They called the resulting values MTD* parameters.



All of the compounds can be covered by the hypermolecule. None of the positions is superfluous.

Compound	Positions
H	1
2-Cl	1 2
2,6-F_2; 2,6-Cl_2; 2,6-Br_2; 2,6-I_2	1 2 3
2-F,6-Cl; 2-Cl,6-OH; 2,6-$(CH_3)_2$	1 2 3
2-Cl,6-Br; 2-Cl,6-CH_3	1 2 3
3-Cl	1 8
2-OCH_3	1 2 4
2-CF_3,6-Cl	1 2 3 4 5 6
2-OC_2H_5,6-Cl	1 2 3 4 10
2,6-$(OCH_3)_2$	1 2 3 4 7
2,6-Cl_2,3-OCH_3	1 2 3 8 10
2,6-Cl_2,3-$OCOCH_3$	1 2 3 4 8 10 11
2,3,6-Cl_3	1 2 3 8
2,3,5,6-Cl_4	1 2 3 8 9
2,5-$(OC_2H_5)_2$	1 2 4 7.9 10 12

Figure 1.4 Atomic positions in the hypermolecule of some substituted benzonitriles (Reference 40).

1.4 REGRESSION ANALYSIS

Multiple regression analysis is statistical calculation based on the method of least squares. It is best done with a computer. The validity of the equations and the relative importance of the different parameters are

judged by statistical criteria. These are the multiple correlation coefficient (r^2), the **F** value, the **t** value, and the standard deviation (**s**). A brief description of the criteria follows. For more information, refer to the literature [41].

The multiple correlation coefficient (r^2) is the degree of correspondence between the biological data that were obtained in experiments and those that were calculated by regression analysis, where $r^2 = 1.0$ is a perfect fit. This coefficient, which is often used without the other criteria, is, in fact, the least reliable estimate of "goodness of fit". Its value is that fraction of the sum of the squares of the deviations of observed responses from mean responses (in other words, the variance in the data) that is attributable to the regression equation.

The **F** value is the decision statistic of the **F** test of significance. It indicates whether the correlation of the equation found is significant. When **n** = number of data and **k** = number of independent variables, the **F** value must be checked in a table of distribution values under **k** and **n** - **k** - 1 degrees of freedom [42]. If the **F** value from the regression equation is greater than the critical **F** value in the table, the correlation is significant.

The **t** value of the regression coefficients a, b, c, d, and e (Equation 1.1) is a measure of how much the corresponding parameters add to the correlation. A specific parameter adds significantly to the correlation only when the **t** value of the corresponding parameter is greater than the critical **t** value associated with **n** - **k** - 1 degrees of freedom; confidence intervals of 90 or 95 % can be calculated instead of **t** values.

The standard deviation (**s**) indicates how closely the biological data obtained in experiments correspond with those calculated by regression analysis.

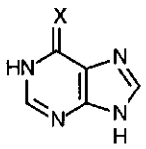
As a rule, a stepwise method is best in regression analysis. Such a method allows one to add the parameters while observing the change in the statistical criteria. Moreover, as it is important to check for intercorrelations between supposedly independent variables, a matrix of the coefficients of all possible intercorrelations will have to be made. Intercorrelated parameters are permissible only if a significant decrease in the standard deviation has been obtained. Despite this, checks for possible intercorrelations are not always done in QSAR studies.

1.5 EXAMPLES

Numerous QSAR studies have focused on the inhibition of certain enzymes. Two of these enzymes are of interest here, namely xanthine oxidase and dihydrofolate reductase. A more detailed treatment of xanthine oxidase follows in the remaining chapters of this thesis.

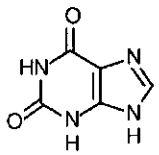
1.5.1 Xanthine Oxidase

Xanthine oxidase converts hypoxanthine (**1.1a**) to xanthine (**1.2**) and then to uric acid (**1.3a**). A high production of uric acid leads to a high concentration of sodium urate in the extracellular fluids. Because of its relative insolubility, sodium urate builds up in subcutaneous sites such as joints, ultimately causing gout.

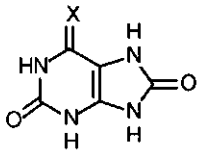


1.1

a: X = O
b: X = S



1.2



1.3

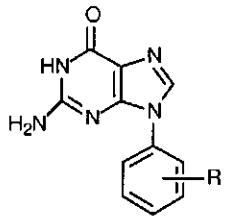
a: X = O
b: X = S

Xanthine oxidase also converts 6-mercaptopurine (**1.1b**), an antileukemic drug, to thiouric acid (**1.3b**). The selective action of 6-mercaptopurine on tumor cells has been linked to the absence or low concentration of xanthine oxidase in those cells. Accordingly, one can assume that tumor cell lines, which are unresponsive to 6-mercaptopurine, contain high levels of xanthine oxidase and that selective inhibition of xanthine oxidase is useful in 6-mercaptopurine therapy.

Baker and co-workers [43] synthesized an extensive series of xanthine oxidase inhibitors and measured the biological activity. Silipo and Hansch [44] compiled Baker's data and made a QSAR study of derivatives of 9-(R-phenyl)guanines (**1.4-1.69**, Table 1.2).

The QSAR study by Silipo and Hansch revealed a correlation between the inhibitory activity of the compounds, the molar refractivity, and the Taft steric parameters:

Table 1.2 Inhibitors of Xanthine Oxidase and Their Inhibition Activity



Compound	R[a)]	-Log I_{50}[b)]	Compound	R[a)]	-Log I_{50}[b)]
1.4	2-Cl	5.09	**1.37**	4-$O(CH_2)_2$X-4'-SO_2F	6.48
1.5	2-Br	5.11	**1.38**	4-Y-3'-SO_2F	6.55
1.6	3-X-3'-SO_2F, 4-OCH_3	5.25	**1.39**	3-Cl	6.57
1.7	3-Y-3'-SO_2F, 4-OCH_3	5.31	**1.40**	$CH(CH_3)_2$	6.60
1.8	3-X-4'-SO_2F, 4-OCH_3	5.35	**1.41**	4-C_6H_5	6.60
1.9	2,3-CH=CHCH=CH	5.38	**1.42**	3-CH_3	6.62
1.10	3-Y-4'-SO_2F, 4-OCH_3	5.39	**1.43**	3-NHCHO	6.64
1.11	4-Z-4'-SO_2F	5.60	**1.44**	3-4-OCH_3	6.66
1.12	4-$NH(CH_3)_2$	5.68	**1.45**	4-OH	6.68
1.13	4-$NHCOCH_2Br$	5.72	**1.46**	4-$O(CH_2)_2$X-3'-SO_2F	6.74
1.14	4-Cl	5.74	**1.47**	3-CF_3	6.82
1.15	4-$C(CH_3)_3$	5.74	**1.48**	4-$O(CH_2)_2$X'-4'-CH_3, 3'-SO_2F	6.92
1.16	4-CH_3	5.80	**1.49**	3-Y-3'-SO_2F	6.96
1.17	4-CF_3	5.89	**1.50**	4-OC_2H_5	6.96
1.18	3,4-Cl_2	5.96	**1.51**	3-$NHCOCH_2OC_6H_4$-4'SO_2F	7.00
1.19	4-$O(CH_2)_3$X-4'-SO_2F	6.00	**1.52**	4-$O(CH_2)_2$X'-2'-Cl, 5'-SO_2F	7.04
1.20	4-Z-3'-SO_2F	6.02	**1.53**	3-Y'-4'-CH_3, 3-SO_2F	7.04
1.21	3,4-$(OCH_3)_2$	6.14	**1.54**	4-$O(CH_2)_3C_6H_5$	7.08
1.22	4-Y-4'-SO_2F	6.15	**1.55**	3-C_6H_5	7.09
1.23	4-$O(CH_2)_2$Y-4'-SO_2F	6.16	**1.56**	3-$NHCOC_6H_5$	7.14
1.24	4-$O(CH_2)_3$X-4'-SO_2F	6.16	**1.57**	3-$NHCOCH_2Br$	7.15
1.25	4-C_2H_5	6.17	**1.58**	3-Y'-2'-Cl, 5'-SO_2F	7.15
1.26	4-$O(CH_2)_3$Y-3'-SO_2F	6.20	**1.59**	4-$O(CH_2)_2$-X'-2'-OCH_3,5'-SO_2F	7.16
1.27	2-F	6.21	**1.60**	3-X'-2'-Cl, 5'-SO_2F	7.28
1.28	4-$(CH_2)_3CH_3$	6.21	**1.61**	3-Y-4'-SO_2F	7.29
1.29	3-NH_2	6.22	**1.62**	3-X'-3'-Cl, 4'-SO_2F	7.48
1.30	4-$O(CH_2)_2$Y-3'-SO_2F	6.28	**1.64**	4-$NHCO(CH_2)_2C_6H_4$-4'-SO_2F	7.58
1.31	4-OCH_3	6.30	**1.65**	3-X-4'-SO_2F	7.62
1.32	4-$O(CH_2)_2$Y'-4'-CH_3, 3'-SO_2F	6.31	**1.66**	3-X'-4'-CH_3, 3'-SO_2F	7.74
1.33	4-$CONH_2$	6.38	**1.67**	3-X'-2'-OCH_3, 5'-SO_2F	7.80
1.34	3,4-CH=CHCH=CH	6.39	**1.68**	3-$NHCOCH_2C_6H_4$-4'-SO_2F	7.82
1.35	H	6.39	**1.69**	3-$NHCO(CH_2)_4C_6H_4$-4'-SO_2F	8.00
1.36	4-$O(CH_2)_3$X-3'-SO_2F	6.40			

a) X = $NHCONHC_6H_4$, Y = $NHCOC_6H_4$, Z = $NHSO_2C_6H_4$, X' = $NHCONHC_6H_3$, Y' = $NHCOC_6H_3$.

b) -Log I_{50} values were taken from Reference 42.

$$\log 1/I_{50} = 0.267(\pm0.06)MR^3 - 0.647(\pm0.12)\{MR^3*MR^4\} + 1.291(\pm0.39)E_S^2 + 0.101(\pm0.04)MR^4 + 0.252(\pm0.11)E_S^4 + 4.552(\pm0.45) \quad (1.6a)$$

$n = 65 \qquad r = 0.910 \qquad s = 0.308$

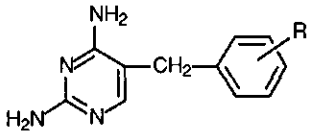
The superscript of the parameters shows the position of the substituent (R). The positive coefficient of MR^3 indicates a positive interaction for bulky substituents at position 3. The negative coefficient of the cross-product term, MR^3*MR^4, shows that bulky substituents at positions 3 and 4 substantially reduce the inhibitory activity, suggesting that the substituent space near these positions is limited. This is caused primarily by the substituent at position 4. The coefficient of MR^4 is small and the positive coefficient of E_S^4 indicates a steric hindrance at that position (the bulkier the substituent, the more negative the value of E_S). Moreover, the high coefficient for E_S^2 indicates a strong steric hindrance at position 2.

Recently, Folkers [45] used computer-aided molecular graphics to model the interaction of some derivatives of 7-phenylpyrolo-[2,3-d]pyrimidine with some model receptors of the active site in xanthine oxidase. His results were disappointing and further research is needed.

1.5.2 Dihydrofolate Reductase

Dihydrofolate reductase (DHFR) catalyzes the reduction of dihydrofolate to tetrahydrofolate, a substance only one step short of the coenzyme for thymine synthesis. The design of DHFR inhibitors is an important goal of research on antibacterial and antitumor drugs. A recent exhaustive review of the QSAR of DHFR inhibitors [46] showed that a wide variety of compounds had been tested and that, consequently, an equal number of QSAR's had been generated. Depending on the source of the enzyme and the type of the inhibitor, several parameters (steric, electronic, and hydrophobic) had produced a relevant correlation, but hydrophobic parameters were dominant.

Table 1.3 Inhibitors of Chicken Liver Dihydrofolate Reductase and Their Inhibition Activity



Compound	R	-Log K_i[a)]	Compound	R	-Log K_i[a)]
1.70	3,4,5-$(CH_2CH_3)_3$	5.25	**1.97**	3-CH_3	4.72
1.71	3,5-$(OCH_3)_2$, 4-$(OCH_2CH_2OCH_3)$	3.64	**1.98**	3-Cl	5.01
1.72	3,4,5-$(OCH_3)_3$	3.98	**1.99**	3-Br	5.03
1.73	3,5-$(OCH_3)_2$, 4-$N(CH_3)_2$	4.15	**1.100**	3-CF_3	4.92
1.74	3,5-$(OCH_3)_2$, 4-Br	4.54	**1.101**	3-$CH_2O(CH_2)_3CH_3$	5.17
1.75	3,5-$(OCH_3)_2$, 4-SCH_3	4.29	**1.102**	3-I	4.79
1.76	3,5-$(OCH_3)_2$, 4-$C(CH_3)=CH_2$	4.17	**1.103**	3-$O(CH_2)_3CH_3$	5.22
1.77	3,5-$(OCH_2CH_3)_2$, 4-C_4H_4N	4.33	**1.104**	3-$OCH_2C_6H_5$	5.63
1.78	3,5-$(CH_2OH)_2$	3.23	**1.105**	3-$O(CH_2)_5CH_3$	5.67
1.79	3,5-$(OCH_3)_2$	4.12	**1.106**	3-$O(CH_2)_6CH_3$	4.79
1.80	3,5-$(OCH_2CH_3)_2$	4.14	**1.107**	3-$O(CH_2)_7CH_3$	5.08
1.81	3,5-$(CH_3)_2$	4.16	**1.108**	4-NH_2	3.73
1.82	3,4-$(OH)_2$	3.59	**1.109**	4-$NHCOCH_3$	4.26
1.83	3-NO_2, 4-$NHCOCH_3$	4.34	**1.110**	4-$OCH_2CH_2OCH_3$	4.26
1.84	3,4-$(OCH_2CH_2OCH_3)_2$	3.91	**1.111**	4-NO_2	4.37
1.85	3,4-OCH_2O-	4.68	**1.112**	4-OCH_3	4.29
1.86	3,4-$(OCH_3)_2$	4.46	**1.113**	4-F	4.79
1.87	3-CF_3, 4-OCH_3	4.99	**1.114**	4-CH_3	4.56
1.88	3-$O(CH_2)_7CH_3$, 4-OCH_3	4.71	**1.115**	4-Cl	4.83
1.89	3-OCH_2CONH_2	4.27	**1.116**	4-Br	4.79
1.90	3-CH_2OH	4.31	**1.117**	4-OCH_3	4.32
1.91	3-OSO_2CH_3	4.33	**1.118**	4-$O(CH_2)_3CH_3$	4.67
1.92	3-CH_2OCH_3	4.37	**1.119**	4-$OCH_2C_6H_5$	4.83
1.93	3-OH	3.87	**1.120**	4-$O(CH_2)_5CH_3$	4.71
1.94	3-$OCH_2CH_2OCH_3$	4.83	**1.121**	4-$O(CH_2)_6CH_3$	4.73
1.95	3-OCH_3	4.45	**1.122**	H	4.71
1.96	3-F	4.70			

a) -Log K_i values were taken from Reference 45.

Fortunately, the X-ray structure of DHFR is known, which made it possible for Selassie et al. [47] to correlate a QSAR of several benzylpyrimidine analogues (**1.70-1.122**, Table 1.3) with molecular-graphics models constructed from X-ray crystallographic coordinates of trimethoprim (**1.72**) and 5-(3,5-dimethoxy,4-isopropenylbenzyl)-2,4-diamino-pyrimidine (**1.76**) bound to DHFR (Table 1.3). This QSAR indicates that bulky substituents have a favorable effect at position 3, as shown below by the positive coefficient for MR^3 and π^3:

$$\begin{aligned} \text{Log } 1/K_i = {} & 0.43(\pm 0.11)\,\pi^3 - 1.13(\pm 0.35)(\log(\beta^3 * 10^{\pi^3} + 1) \\ & - 0.66(\pm 0.19)MR^5 + 0.59(\pm 0.22)\pi^4 + 0.48(\pm 0.30)\pi^5 \\ & - 0.63(\pm 0.33)\log(\beta^4 * 10^{\pi^4} + 1) + \\ & 0.14(\pm 0.09)MR^3 + 4.64(\pm 0.14) \end{aligned} \qquad (1.6b)$$

$\mathbf{n} = 53$ $\quad \mathbf{r} = 0.921$ $\quad \mathbf{s} = 0.208$ $\quad \mathbf{F} = 8.07$

$\log\beta^3 = -2.21$ $\quad \log\beta^4 = -0.07$

For the **meta**-substituted compounds, a single substituent is called 3. When two substituents are present, they are called 3 and 5. At position 5, there seems to be a strong steric hindrance to binding, which offsets the smaller positive hydrophobic effect indicated by π^5. Thus the binding at position 5 seems to occur in a sterically limited hydrophobic space. At positions 3 and 4, the substituents seem to have an optimum hydrophobicity value.

After calculating Equation 1.6b, Selassie et al. viewed the possible interactions of the inhibitors with DHFR using molecular graphics based on X-ray crystallographic data of DHFR and molecular models of the compounds. The agreement was remarkable. At position 4, for example, it appears that, in the aqueous phase, substituents larger than 4-*n*-butoxy will meet a polar area and eventually protrude beyond the enzyme space. Similarly, a long hydrophobic substituent at position 3 can bind very well up to a value of $\pi = 2.00$. Beyond this point, steric hindrance occurs owing to blockage of the substituent by a proline residue from the enzyme.

Selassie et al. show very clearly how QSAR (which indicates possible interactions and at which positions) and molecular graphics (which give a more detailed picture of the interactions) can complement each other.

1.6 OUTLINE OF THIS THESIS

Xanthine oxidase is one of the first enzymes for which a highly purified preparation was obtained [48]. Because it is easy to isolate, well-documented, and commercially available, it has become a common subject of research for biochemists and medicinal chemists.

The goal of this thesis is to shed more light on the structural requirements for binding organic compounds with xanthine oxidase. Chapter 2 contains a description of the synthesis of the organic compounds used for this thesis.

Chapters 3 and 5 contain the results of inhibition studies of two different congeneric compound groups and a discussion of a structure-activity relationship for both.

Chapter 4 contains a discussion of the oxidation of hypoxanthine analogues by xanthine oxidase.

Chapter 6 contains general results and a general discussion of the structure of the active site. The results and the discussion are based on research that was done for this thesis and on previous work of this University's Department of Organic Chemistry.

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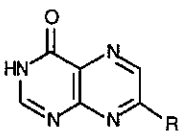
2 SYNTHESIS OF THE STARTING COMPOUNDS

2.1 INTRODUCTION

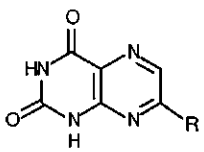
Tramper et al. [1] have established that immobilized xanthine oxidase is an effective reagent for oxidizing the pteridin-4-ones (**2.1a**) and their 7-alkyl (**2.1b**) and 7-aryl (**2.1c**) derivatives into the corresponding lumazines (**2.2**). The 7-arylpteridin-4-ones (**2.1c**) are especially good substrates. Their K_m value is lower than that of xanthine (**2.3a**), the pteridin-4-ones, and the alkylpteridin-4-ones, indicating that the 7-aryl compounds are bound more strongly to the enzyme [1b]. This is probably due to an interaction of the aryl group with a hydrophobic region at the active site of xanthine oxidase. Researchers have observed such an interaction with 9-arylpurines [2], 3-aryl-5,7-dihydroxypyrazolo[1,5-a]pyrimidines [3a], and 2-aryl-4-trifluoro-methylimidazoles [3b].

The 6-phenylpteridin-4-ones behave quite differently. They are very poor substrates for xanthine oxidase, effectively inhibiting it in the oxidation of xanthine and 7-arylpteridin-4-ones [1b].



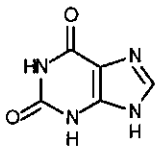
2.1

a: R = H
b: R = Alkyl
c: R = Aryl



2.2

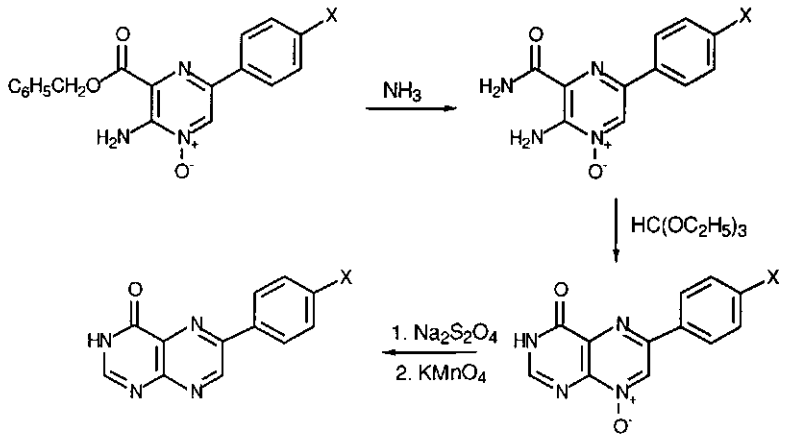
a: R = H
b: R = Alkyl
c: R = Aryl



2.3

Continuing interest in new, more effective, and more specific xanthine oxidase inhibitors [3] has prompted more detailed investigations of the inhibitory properties of the 6-arylpteridin-4-ones (**2.6**) and the 8-arylhypoxanthines (**2.9**). A description of some synthetic methods of obtaining these compounds follows.

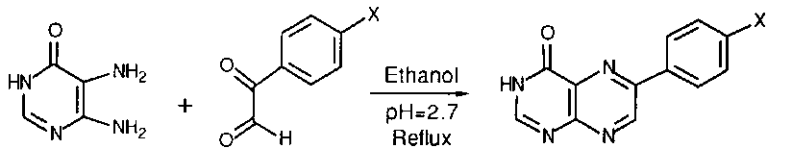
There are two synthetic methods of obtaining 6-substituted pteridines, namely the Taylor method [4] and the Gabriël-Isay method [6]. The Taylor method is the most recent. De Meester et al. [5] have used it to synthesize several 6-arylpteridin-4-ones (Scheme 2.1).



Scheme 2.1

The Taylor method is very elegant, for the pteridines obtained with it are not contaminated by isomeric substituted pteridines, as are those obtained with the Gabriël-Isay method. Nevertheless, the Taylor method involves four reaction steps, and its final yield is relatively low. Moreover, using this method, De Meester et al. [5b] were able to synthesize **2.6** with only five different aryl groups.

A more general way to obtain pteridines is with the Gabriël-Isay method, in which a 4,5-diaminopyrimydine reacts with a 1,2-dicarbonyl compound (Scheme 2.2).



2.4 **2.5** **2.6**

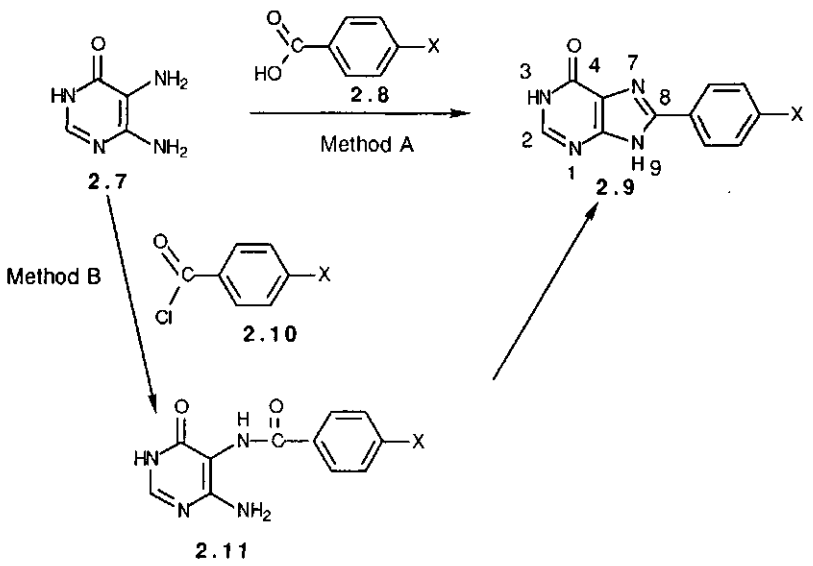
+ 7-phenyl isomer (**2.1c**)

Scheme 2.2

For pteridin-4-ones, when an aryl group substitutes for the hydrogen at position 6, this reaction requires 4,5-diaminopyrimydin-6-one (**2.4**) and an arylglyoxal (**2.5**). The reaction, however, usually produces a mixture of 6-aryl and 7-aryl substituted pteridin-4-ones [7]. The acidity of the reaction medium influences the ratio of the two products, so that the 7-arylpteridin-4-ones (**2.1c**) thus obtained are almost pure when the reaction is done at pH 7.5. The 6-arylpteridin-4-ones (**2.6**) are obtained with a more acidic medium [7a-b].

For this thesis, the Gabriël-Isay method was preferable, as it involves only one step, and the problem of separating the isomeric 6-arylpteridin-4-ones and 7-arylpteridin-4-ones was solved in the course of the research.

The 8-arylhypoxanthines (**2.9**) were synthesized with starting compounds similar to those that were used to synthesize the pteridines. These synthetic procedures are outlined in Scheme 2.3. The first series of 8-arylhypoxanthines was synthesized according to a method developed by Garmaise and Komlossy [8] (method A). Later series were synthesized according to a method proposed by Fu et al. [9] (method B), which extends the congeneric series.



Scheme 2.3

2.2 MATERIALS AND METHODS

2.2.1 Materials

The **para**-substituted benzoic acids and several of the **para**-substituted acetophenones used for the research presented in this thesis were bought from Aldrich. The acetophenone derivatives with X = iodo, ethyl, *n*-propyl, *i*-propyl, *t*-butyl, and *n*-butyl were easily synthesized with a Friedel-Crafts acetylation of the appropriate benzene derivatives. The substituted phenylglyoxals were obtained with published procedures [10]. Melting points were measured on a Kofler hot plate. They are presented here uncorrected.

The ^{1}H-NMR spectra were recorded in deuterated dimethylsulfoxide (DMSO-d_6) on either a Hitachi Perkin-Elmer R24B spectrometer, a Varian EM-390 spectrometer, or a Bruker CXP-300 spectrometer operating in the Fourier transform mode, with tetramethylsilane as an internal standard (δ = 0 ppm). The ^{13}C-NMR spectra were recorded in the same solution (DMSO-d_6) on either a Varian XL-100 spectrometer or a Bruker CXP-300 spectrometer operating in the Fourier transform mode and employing the solvent peak as a standard (δ = 39.6 ppm).

2.2.2 Synthesis of the 6-(pX-Phenyl)Pteridin-4-Ones(**2.6a-m**)

A solution of 3.5 g (22.7 mmole) of 4,5-diaminopyrimidin-6-one (**2.4**) in 50 ml of 96% ethanol was acidified with diluted sulphuric acid to pH 2.7 at 60^{o}C. Subsequently, 27 mmole of compound (**2.5**), dissolved in 20 ml of 96% ethanol, was stirred into the solution. This reaction mixture was then refluxed. The pH was kept at 2.7 with the addition of 0.5 N NaOH. When the pH had remained constant for approximately two hours, the mixture was allowed to cool.

The crystals obtained from the reaction were then filtrated and washed with ethanol and ether. The crude product (yield 70 to 90%), which contained both the 6-aryl and 7-aryl isomers of pteridin-4-one, was fractionated by recrystallization from DMSO. The 7-isomer crystallized first. Finally, an equal volume of water was added to the filtrate to precipitate the 6-isomer and residual 7-isomer. This procedure was repeated until the 6-isomer was free from any 7-isomer, as judged by ^{1}H-NMR.

An alternative recrystallization method was possible for compounds **2.6g-j** (Table 2.1). In this method, the crude product of the reaction mixture was washed extensively with hot methanol. The residue was the 7-aryl isomer. The methanol filtrates were then combined, concentrated by vacuum-evaporation, and cooled until they crystallized. This fraction contained mainly the 6-isomer. Recrystallization from methanol yielded the pure compounds, as judged by ^{1}H-NMR.

2.2.3 Synthesis of the 8-(pX-Phenyl)Hypoxanthines(**2.9a-k**)

With Method A [9]

Equal molar amounts (30 mmole) of 4,5-diaminopyrimidin-6-one and the appropriate benzoic acid were mixed in 30 ml of freshly prepared polyphosphoric acid. The reaction mixture was heated to 190°C, stirred continuously at that temperature for two hours, and allowed to cool. The resulting dark-brown solution was diluted with 200 ml of water and filtrated.

The filtrate was then neutralized with a solution of 25% NaOH and the resulting precipitate was filtrated. The crude product was dissolved in 1 N NaOH, norit was added, and the solution was refluxed for ten minutes. The hot solution was filtrated and the filtrate was acidified with acetic acid.

The filtrate was then cooled to 4°C and the resulting precipitate was collected by filtration. The crude product was purified by an additional base-acid precipitation and washed with alcohol and ether. The final product was dried *in vacuo* with phosphorus pentoxide.

With Method B [10]

To prepare the substituted benzoyl chloride, 15 g of the appropriately substituted benzoic acid were dissolved in 100 ml of $SOCl_2$. The resulting mixture was refluxed for one hour and the excess $SOCl_2$ was evaporated *in vacuo*. The remaining liquid was used for the next step without being purified further.

The benzoyl chloride was then cooled to 0°C and mixed with 10 g of 4,5-diaminopyrimidin-6-one that had been dissolved in 100 ml of 1 N NaOH. The mixture was stirred at 0°C for forty-eight hours. The resulting turbid solution was made more alkaline with the addition of some solid NaOH. The solution was extracted with ether, whereupon the aqueous layer

became clear. This basic solution was acidified to pH 5 with 6 N HCl. The resulting precipitate was collected by filtration and washed with water and ether.

The 4-amino-5-arylamidopyrimidin-6-one (**2.11**) was then purified. This was done by dissolving it in 0.5 N NaOH and refluxing it with norit for ten minutes. The norit was filtered off and the filtrate was acidified to pH 6 with 4 N HCl. The resulting precipitate was filtered, washed with water and ether, and dried *in vacuo* with phosphorus pentoxide.

The ring closure of 4-amino-5-arylamidopyrimidin-6-one (**2.11**) was then accomplished. This was done by mixing in a flask 15 mmole of the compound with 25 g of phosphorus pentoxide. The flask was cooled in ice and 18 ml of 85% polyphosphoric acid were added. The reaction mixture was heated to 180°C and kept at that temperature for four hours, after which the hot solution was poured over crushed ice. This solution was neutralized to pH 5 with 6 N NaOH and the resulting precipitate was filtered off.

The crude product was then dissolved in 1 N NaOH and refluxed with norit for ten minutes. The norit was removed and the filtrate was acidified to pH 6. The resulting precipitate was collected by filtration, washed with water and ether, and dried *in vacuo* with phosphorus pentoxide.

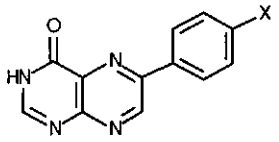
2.3 RESULTS AND DISCUSSION

2.3.1 Synthesis of the 6-Arylpteridin-4-Ones

The 6-arylpteridin-4-ones (**2.6a-m**) used for this thesis were synthesized by condensing 4,5-diaminopyrimidin-6-one (**2.4**) with an arylglyoxal derivative (**2.5**) according to published procedure [7a]. The results are shown in Table 2.1. The product is usually a mixture of 6-arylpteridin-4-one and the 7-substituted isomer (**2.1c**). Nevertheless, the composition of the reaction mixture depends on the pH at which the reaction is carried out. At pH 2.7, the main product is **2.6** (Table 2.2).

To obtain pure **2.6**, the reaction products were recrystallized from dimethylsulfoxide (DMSO) or from methanol, as described in the section on

Table 2.1 Synthesis of the 6-Arylpteridin-4-Ones



No.	X	M. Pt. (°C)	Yield %	Formula	Analysis[a]	^{1}H-NMR[b] H-2	H-7	^{13}C-NMR[c] C-6	C-7
2.6a	H	304[e]	35%	$C_{12}H_8N_4O.H_2O$	C,H	8.35	9.58	149.9	146.7
2.6b	F	322[e]	6%	$C_{12}H_7N_4FO$	C,H	8.35	9.60	149.4	147.5
2.6c	Cl	313[e]	6%	$C_{12}H_7N_4ClO.H_2O$	C,H	8.30	9.55	149.4	147.8
2.6d	Br	315[e]	17%	$C_{12}H_7N_4BrO.H_2O$	C,H	8.30	9.55	149.1	147.5
2.6e	I	332[e]	22%	$C_{12}H_7N_4IO$	C,H	8.35	9.60	149.2	147.2
2.6f	Me	286[e]	23%	$C_{13}H_{10}N_4O.H_2O$	C,H	8.30	9.50	149.5	146.2
2.6g	Et	253-5	25%	$C_{14}H_{12}N_4O$	C,H	8.30	9.56	150.3	147.5
2.6h	*n*-Pr	255-8	25%	$C_{15}H_{14}N_4O$	C,H	8.30	9.55	149.4	147.3
2.6i	*i*-Pr	233[e]	20%	$C_{15}H_{14}N_4O$	C,H	8.30	9.52	150.3	147.4
2.6j	*t*-Bu	265[e]	15%	$C_{16}H_{16}N_4O$	C,H	8.33	9.53	150.2	147.4
2.6k	OCH_3	280[e]	15%	$C_{13}H_{10}N_4O_2.H_2O$	C,H	8.30	9.55	149.1	145.9
2.6l	*n*-Bu	260[e]	5%	$C_{16}H_{16}N_4O$	C,H	8.33	9.60	150.6	147.3
2.6m	OH	340	10%	$C_{12}H_8N_4O_2$	C,H[f]	8.30	9.50	149.3	146.8
Pteridine-4-one[d]				$C_6H_4N_4O$				144.2	150.2
7-Phenyl-Pteridine-4-one		345	75%	$C_{12}H_8N_4O$	C,H	8.30	9.40[g]	141.9	155.7

a) Micro-analyses for C and H were within 0.4% of the calculated theoretical value.
b) Chemical shifts in .δ (ppm).
c) Chemical shifts in ppm.
d) A. Nagel, unpublished results.
e) Decomposes upon melting.
f) Analytical calculation for $C_{12}H_8N_4O_2.{}^1/_2H_2O$: C, 57.83; H, 3.64; Found: C, 58.48; H, 3.33.
g) Chemical shift for H-6.

Table 2.2 Effect of pH on Isomer Formation in the Reaction of **2.4** and **2.5**

pH	6-Isomer **2.6a**	7-Isomer **2.1c**
7.5	<5%	>95%
4.5	70%	30%
3.5	70%	30%
2.7	75%	25%
2.0	60%	40%

materials and methods. The purity of **2.6** was established by ^{1}H-NMR spectroscopy, which revealed the absence of the H-6 absorbance at δ = 9.4 ppm of the 7-arylpteridin-4-one. With the Varian EM-390 spectrometer, it was possible to detect impurities as slight as 5% in the 6-arylpteridin-4-one samples.

Both ^{1}H-NMR and ^{13}C-NMR spectroscopy provided solid evidence that the aryl group is indeed in position 6. The structure assignment given in this thesis is based mainly on ^{13}C-NMR spectroscopy.

While the aryl group shifts the absorption of the carbon atom to which it is attached 5 to 6 ppm downfield, it shifts its neighboring carbon atom 4 to 5 ppm upfield [11]. Consequently, relative to the C-6 and C-7 signals of pteridin-4-one, the separation between the corresponding ^{13}C-NMR signals in the 6-arylpteridin-4-ones (3 to 4 ppm) is much smaller than the separation between the signals in the 7-phenylpteridin-4-one (13 to 14 ppm).

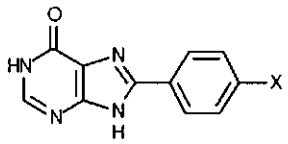
Thirteen 6-arylpteridin-4-ones were synthesized for this thesis with the Gabriël-Isay method. In these syntheses, the method showed one minor disadvantage, namely the occasional presence in the final product of minute amounts of the isomeric 7-arylpteridin-4-ones. One way to avoid this is to synthesize the 6-arylpteridin-4-ones from the corresponding pyrazine derivatives. The key intermediate in this synthesis is then 2-amino-3-benzyloxycarbonyl-5-arylpyrazine-1-oxide, as De Meester et al. [5b] showed (Scheme 2.1). Although they were able to synthesize many different 2-amino-3-benzyloxycarbonyl-5-arylpyrazine-1-oxides, De Meester et al. were not able to effect subsequent closure of the pyrimidine ring.

2.3.2 Synthesis of the 8-Arylhypoxanthines

The 8-arylhypoxanthines used for this thesis were synthesized with either method A or method B (Table 2.3). With method A, an initial series of seven compounds was synthesized(**2.9a** and **b**, and **g-k**). Fortunately, when no more compounds could be obtained with method A, it was still possible to synthesize four more compounds (**29c-f**) with method B.

With method B, the 4,5-diaminopyrimidin-6-one (**2.7**) reacted first with a **para**-substituted benzoylchloride (**2.9**) to form 4-amino-5-arylamidopyrimidin-6-one (**2.11**). The ring closure of **2.11** to purine (**2.9**)

Table 2.3 Synthesis of the 8-Arylhypoxanthines



No.	X	Method[a)]	Yield %	Analysis	M.Pt (°C)	m/e Calc.	m/e Meas.	pKa[c)]
2.9a	H	A	60%	C,H,N	320 [b)]	212.0698	212.0692	8.6
2.9b	CH_3	A	59%		>330	226.0855	226.0855	8.6
2.9c	*i*-C_3H_7	B	52%		>330	254.1168	254.1170	
2.9d	*n*-C_4H_9	B	54%	C,H,N	>330	268.1324	268.1313	
2.9e	*t*-C_4H_9	B	60%	C,H,N	>330	268.1324	268.1322	
2.9f	F	B	65%		>330	230.0604	230.0606	
2.9g	Cl	A	62%		>330	246.0308	246.0306	8.5
2.9h	Br	A	61%	C,H,N	>330	289.9804	289.9811	8.9
2.9i	NO_2	A	53%	C,H,N	315 [b)]	decomposes		7.5
2.9j	$N(CH_3)_2$	A	48%	C,H,N	308 [b)]	255.1120	255.1109	10.2
2.9k	OCH_3	A	37%	C,H,N	270 [b)]	242.0804	242.0799	8.8

a)For details of methods A and B, see Materials and Methods.

b)Decomposes upon melting.

c)The pKa values were measured according to Albert and Sergeant [12].

was achieved with polyphosphoric acid. Method B was especially suitable for synthesizing the alkyl-substituted 8-arylhypoxanthines.

Attempts to extend this series of 8-arylhypoxanthines with larger alkyl or alkoxy groups, or with other substituents such as X = pentyl, ethoxy, phenyl, or cyano, were unsuccessful. Although it was relatively easy to form 4-amino-5-arylamidopyrimidin-6-one (**2.11**), it was impossible to achieve ring closure. To surmount this obstacle, the reaction conditions for closure of the imidazole ring were varied. The compounds were refluxed in a solution of 30% KOH [13], in acetic acid anhydride [14], in $POCl_3$ [15], in absolute ethanol [16], and by sublimation [15]. None of these measures helped, doubtless because the carbonyl group is somehow deactivated by the substituents. Consequently, for this series, the scope of this thesis is limited to eleven compounds, namely **2.9a-k**.

The characterization of these compounds was done with ^{1}H-NMR spectroscopy, elemental analysis, and molecular weight calculations from

the mass spectra. Between 6.8 and 8.1 ppm, the ^{1}H-NMR spectra showed the normal AB pattern of the aromatic protons from the aryl group. The characteristic absorption of the H-2 around 8.0 ppm was also evident. As might have been expected, measurements showed that, except in the nitro and the dimethylamino-substituted compounds, the pKa values do not vary greatly.

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3 QSAR ANALYSIS OF THE INHIBITION OF XANTHINE OXIDASE BY 6-ARYLPTERIDIN-4-ONES [1]

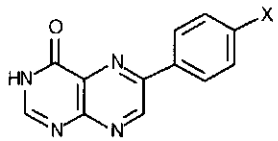
3.1 INTRODUCTION

Xanthine oxidase has three prosthetic groups, namely flavine adenine dinucleotide (FAD), molybdenum, and an iron-sulfur cluster. For the most part, the mechanism of the electron transfer sequence (from the substrate, through the prosthetic groups, towards oxygen) is resolved. The molecular weight of xanthine oxidase, however, is high (275,000 to 300,000 daltons), making X-ray studies difficult [2], and extended X-ray absorption fine-structure spectroscopy (EXAFS) studies of the structure of its molybdenum centers have only been partially successful [3].

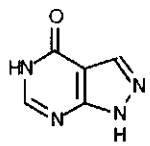
Xanthine oxidase is an effective reagent for oxidizing the pteridin-4-ones and their 7-alkyl and 7-aryl derivatives into the corresponding lumazines [4]. The 7-arylpteridin-4-ones are especially good substrates [3b].

The 6-arylpteridin-4-ones (**3.1**) behave quite differently. Contrary to their 7-isomeric compound, they are not good substrates for xanthine oxidase. Instead, they inhibit the oxidation of xanthine and the 7-aryl-pteridin-4-ones by xanthine oxidase. It is possible that the strong interaction of these arylpteridin-4-ones is due to the interaction of the aryl group with a hydrophobic region at the active site of xanthine oxidase [3b]. A similar strong interaction has been observed with other aryl-substituted inhibitors of xanthine oxidase [5,6].

The search for still more xanthine oxidase inhibitors continues. A primary goal is to find an alternative to allopurinol (**3.2**), which is an analog of hypoxanthine and the most common potent xanthine oxidase inhibitor used to treat gout [7]. Allopurinol has some serious side effects (e.g. leukopenia, dermatitis, rashes, renal impairment, and gastro-intestinal disorders) [8]. Cessation of treatment generally alleviates these symptoms.



3.1



3.2

Like all structural analogs of xanthine, allopurinol is a substrate of phosphoribosyl transferase, by which it is converted to a ribonucleotide with an anti-metabolic potential [9]. Briley and Eisenthal [10a] have reported that allopurinol can also be converted to a nucleoside by purine nucleoside phosphorylase. Although it has not been proved, medical researchers suspect that prolonged use of these compounds could well be harmful.

Most inhibitors studied nowadays are related to purines. In their QSAR analysis of the 9-aryl-purines, Silipo and Hansch [11] found that substituting bulky groups at the **ortho** or **para** position in the phenyl ring diminishes the inhibitor's effectiveness (Section 1.5.1). A primary goal of the research done for this thesis was to evaluate even further the structural requirements for binding to the active site of xanthine oxidase. To do this, special attention was paid to the effects on inhibitory activity of **para**-substitution in the phenyl ring of the 6-arylpteridin-4-ones. In addition, the research included testing of the 6-arylpteridin-4-ones with xanthine oxidase immobilized on *n*-octyl-Sepharose 4B (the reaction product of CNBr-activated Sepharose 4B and *n*-octylamine). This was done to determine whether an immobilized enzyme system might be a better representation of the *in vivo* situation, where xanthine oxidase is associated with membrane systems [10].

3.2 MATERIALS AND METHODS

3.2.1 Materials

The bovine milk xanthine oxidase (E.C. 1.2.3.2) came from Boehringer. The CNBr-activated Sepharose 4B came from Pharmacia. The xanthine came from Fluka and the allopurinol came from Aldrich. The synthesis of the 6-arylpteridin-4-ones is described in Chapter 2.

3.2.2 Enzyme Assays

The xanthine oxidase assay consisted of 2.5 ml of a solution containing 100 mM sodium phosphate buffer (pH 6.9), 0.1 mM EDTA, 25 to 50 μg of xanthine oxidase, 20 to 50 μM of xanthine, and appropriate amounts of the inhibitor. The enzyme assay was done aerobically, at 20°C, at 296 nm

($\delta\varepsilon$ = 10.19 $mM.cm^{-1}$), with an Aminco DW2a spectrophotometer set in the split-beam mode. The absorption was recorded for two to five minutes to measure the initial oxidation rate.

A similar assay was then done for xanthine oxidase immobilized on *n*-octyl-Sepharose 4B. The immobilized xanthine oxidase had been prepared according to a method described by Tramper et al. [12], with which it was possible to immobilize approximately 20 mg of protein/g of Sepharose 4B. This suspension was magnetically stirred in the cuvet while the oxidation rate was measured.

3.3 RESULTS

3.3.1 Inhibition of Xanthine Oxidase

The inhibition constant (K_i) was determined for the free xanthine oxidase and K_i' was determined for the immobilized xanthine oxidase of compounds **3.1a-m** (Table 3.1). This was done by measuring the oxidation rate at five different concentrations of inhibitor and using four different concentrations of the xanthine substrate. The type of inhibition was determined from Lineweaver-Burk plots [13], as Figure 3.1 illustrates for inhibitor **3.1k**. Compound **3.1k** shows a competitive inhibition with the free enzyme (Figure 3.1A) and a mixed-type inhibition with the immobilized enzyme (Figure 3.1B).

The same kinetic analysis was done for all the other compounds. Table 3.1 summarizes the K_i and K_i' values, as determined by Dixon's method [14], and the type of inhibition. A similar kinetic analysis of allopurinol has been included for comparison.

The inhibition parameter of all the compounds is of the same order of magnitude for both the free and immobilized enzyme. The observed trend for the substituent (X) is also similar. Except for compounds **3.1e** and **3.1k**, the apparent K_i' value of the immobilized xanthine oxidase is generally higher than the K_i value of the free xanthine oxidase. These data are insufficient to explain either the difference in the K_i values of the two enzyme systems or the change in the type of inhibition.

Compounds **3.1g** and **3.1h** are mixed-type inhibitors, but the other compounds demonstrate a competitive inhibition for the free enzyme. All

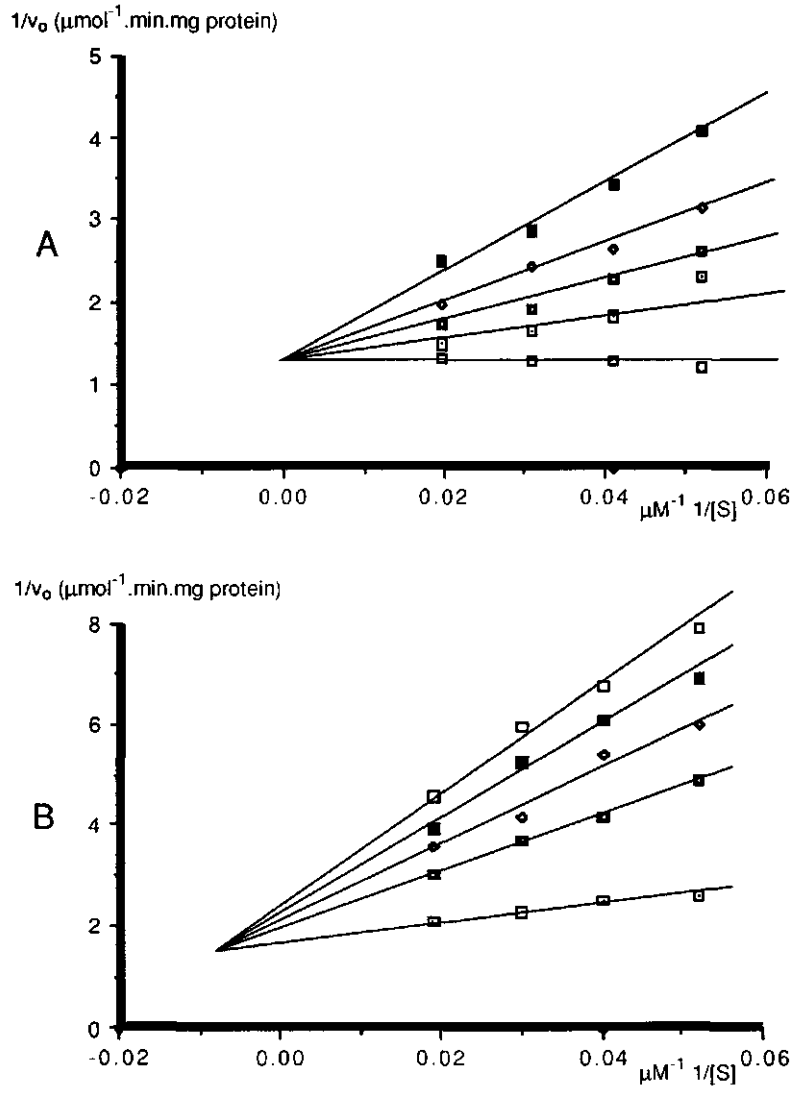


Figure 3.1 Kinetics of inhibition of xanthine oxidase by 6-(**p**OCH_3-phenyl)pteridin-4-one (**3.1k**). The xanthine oxidase assays were performed as described in the section on enzyme assays. In A, the free enzyme system (6.8 µg protein/ml) was used, and the inhibitor concentrations were either 5.19 µM (■-■), 3.11 µM (◇-◇), 1.95 µM (▣-▣), 1.04 µM (□-□), or none at all (□-□). In B, the immobilized enzyme system (18 µg protein/ml, 18.4 mg protein/g Sepharose) was used, and the inhibitor concentrations were either 3.42 µM ((□-□), 2.54 µM (■-■), 1.83 µM (◇-◇), 1.30 µM (■-■), or none at all (▣-▣).

Table 3.1 The K_i Values and Type of Inhibition of Various 6-(pX-Phenyl)Pteridin-4-Ones with Free and Immobilized Xanthine Oxidase

No.	X	Free Xanthine Oxidase		Immobilized Xanthine Oxidase	
		K_i(μM)	Type of Inhibition[a)]	K_i'(μM)	Type of Inhibition[a)]
3.1a	-H	0.17	C	0.45	M
3.1b	-F	0.20	C	0.55	M
3.1c	-Cl	1.9	C	2.2	M
3.1d	-Br	2.5	C	4.5	M
3.1e	-I	3.8	C	2.25	M
3.1f	-methyl	2.4	C	3.0	M
3.1g	-ethyl	1.1	M	1.5	M
3.1h	-*n*-propyl	0.52	M	2.4	M
3.1i	-*i*-propyl	0.50	C	2.5	M
3.1j	-*t*-butyl	0.45	C	1.9	M
3.1k	-methoxy	1.5	C	0.85	M
3.1l	-*n*-butyl	0.15	C	1.4	M
3.1m	-OH	0.30	C	0.45	M
Allopurinol		7.0	M	14.0	M
3.1a	-H[b)]	8.3	NC	11.6	M
Allopurinol[b)]		530	NC	200	C

a)C refers to a competitive inhibition, NC to a noncompetitive inhibiton, and M to a mixed-type inhibition [15].

b)In these assays, 7-phenylpteridin-4-one was used as a substrate ($\delta\varepsilon$(315nm) = 4 $mM.cm^{-1}$).

the compounds exert a mixed-type inhibition in the immobilized enzyme system.

Comparison of the K_i values of the compounds in Table 3.1 shows that the 6-arylpteridin-4-ones inhibit xanthine oxidase much more than allopurinol, and that the 6-aryl substituent strongly influences the interaction of the compounds with xanthine oxidase. These results support previous studies showing that aryl substituents bind the inhibitor more tightly to the hydrophobic region of the enzyme's active site [5, 6]. When the 7-phenylpteridin-4-one is used as the substrate, the same effect occurs and the difference between the K_i values of **3.1a** and those of allopurinol becomes even more pronounced.

3.3.2 QSAR Analysis

A QSAR analysis of xanthine oxidase was done to establish a quantitative relationship between the K_i values and the substituent present in the **para** position of the phenyl group. With the K_i values of the compounds that were prepared at the start of the program, **(3.1a-k)**, the QSAR analysis of the free enzyme system produced the following equation:

$$-\log K_i = -0.795\ B_1\ (t = -3.3) + 0.012\ (MR)^2\ (t = 6.0) - 0.191\ MR\ (t = -5.0) + 1.880 \qquad (3.3)$$

$$n = 11 \quad r = 0.93 \quad s = 0.21 \quad F = 14.3$$

A similar QSAR analysis of the K_i' values of the immobilized enzyme system produced a different equation:

$$-\log K_i' = -0.465\ \pi\ (t = -2.4) + 0.0006\ (MR)^2\ (t = 4.2) - 0.080\ MR\ (t = -2.6) - 0.360\ B_1\ (t = -2.0) + 0.801 \qquad (3.7)$$

$$n = 11 \quad r = 0.93 \quad s = 0.15 \quad F = 9.3$$

In Equations 3.3 and 3.7, B_1 is the minimum van der Waals width, MR is the molar refractivity, and π is the hydrophobic constant of the substituent (X) [16]. The interrelationship of the variables is shown in Table 3.2. The stepwise development of Equation 3.3 is shown in Table 3.3, and that of Equation 3.7 is shown in Table 3.4.

In the regression analysis, neither the Hammett coefficient, B_5 (the maximum van der Waals width), nor L (the van der Waals length) was able to

Table 3.2 Squared-Correlation Matrix Showing Degree of Colinearity (r^2) Between the Variables Used

	π	B_1	MR	$(MR)^2$
π	1.000	0.785	0.921	0.914
B_1		1.000	0.785	0.785
MR			1.000	0.985
$(MR)^2$				1.000

Table 3.3 Stepwise Development of a QSAR Analysis for Free Xanthine Oxidase

Eq.	Intercept	B_1	$(MR)^2$	MR	r	s	F
3.1	0.666	-0.363			0.35	0.45	1.3
3.2	1.398	-1.014	0.003		0.61	0.41	2.3
3.3	1.880	-0.795	0.012	-0.191	0.93	0.21	14.3

Table 3.4 Stepwise Development of a QSAR Analysis for Immobilized Xanthine Oxidase

Eq.	Intercept	π	$(MR)^2$	MR	B_1	r	s	F
3.4	0.040	-0.298				0.63	0.26	6.0
3.5	0.096	-0.760	0.003			0.77	0.23	5.7
3.6	0.358	-0.549	0.006	-0.085		0.88	0.18	7.8
3.7	0.801	-0.465	0.006	-0.080	-0.360	0.93	0.15	9.3

improve the equations. B_1 has a large negative coefficient in both equations, which means that an increase in the minimum van der Waals width will reduce the effectiveness of the inhibitor. MR has an equally negative coefficient, but it also appears as $(MR)^2$, with a small positive coefficient, so, up to a certain value, the increase in the substituent's size will have a negative effect on the inhibitory activity. Nevertheless, if the size of the substituent is increased, the MR part of the equation will eventually have a positive value. In Equation 3.3, this is reached at MR = 7.96. In Equation 3.7, it is reached at MR = 6.66.

The hydrophobicity parameter is necessary to obtain a good analysis for Equation 3.7. This is quite understandable, as in the immobilized enzyme system, one must consider partitioning effects between two phases, namely the bulk solution and the Sepharose matrix in which the enzyme is immobilized. The hydrophobicity parameter (π) is derived from an octanol-water system [16a]. This constant indicates the kind of equilibrium that exists between the two phases. In this study, it indicates the equilibrium between the bulk solution and the Sepharose matrix. In Equation 3.7, π has a large negative coefficient, which means that compounds with polar

substituents (i.e. with a negative π value) will inhibit immobilized xanthine oxidase more effectively than free xanthine oxidase.

3.4 DISCUSSION

The results show that several 6-arylpteridin-4-ones are powerful inhibitors of the oxidation of xanthine by xanthine oxidase. The K_i value of compounds **3.1a** and **3.1l** is about forty times lower than that of allopurinol. If 7-phenylpteridin-4-one is used as a substrate, the K_i value of **3.1a** is sixty times lower than that of allopurinol.

The structural similarity of the 6-arylpteridin-4-ones to substrates like xanthine and 7-phenylpteridin-4-one, combined with the competitive-type inhibition that most compounds displayed with free xanthine oxidase, strongly suggest that these compounds interact with the active site of the enzyme [17]. This would explain the low K_i values obtained for the aryl-substituted inhibitors. The aryl substituent enhances the binding of the compound by interacting with a hydrophobic region in the active site, as Baker et al. [5a-c] reported for aryl-substituted purines.

A QSAR analysis of the 9-arylguanines that Baker et al. synthesized showed that a bulky substituent at the **ortho** and **para** positions in the aryl group reduces the effectiveness of the inhibitor [11]. A QSAR analysis of the data in Table 3.1 not only provided a similar result for some of the compounds, it also gave a good indication of the shape a substituent needs to exhibit good binding properties.

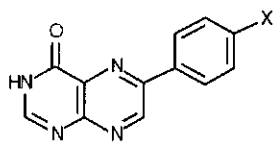
As mentioned previously, Equations 3.3 and 3.7 show that, while the substituent should have some volume, it should not be spherical. A comparison of the K_i values of compounds **3.1e** and **3.1h** demonstrates this. The iodo substituent in Table 3.5 is more spherical, as shown by its large minimum van der Waals width (B_1) The *n*-propyl substituent, while just as "bulky," is more rod-shaped, and thus has a lower K_i value.

The extra parameter in the QSAR analysis of the immobilized enzyme system calls the statistical reliability of the relationship into question, as there are four parameters in Equation 3.7 and only eleven compounds to explain (Section 1.2). The extra parameter (π) can be attributed to partitioning effects. A reasonable assumption is that a bulky substituent will cause a large partitioning effect between the concentrations within the bulk

solution and the support. This effect can be diminished to a certain extent with a polar substituent, which supports the notion that the Sepharose matrix is hydrophilic. Compound **3.1k** is a good example of how to diminish the partitioning effect, for it has an even lower K_i value for the immobilized xanthine oxidase than for the free xanthine oxidase. The other parameters are similar to the ones in Equation 3.3 and explain the interaction of the compounds with the enzyme.

This discussion was tested with two new compounds, namely the *n*-butyl-substituted compound **3.1l** (which has the same MR values as **3.1j**, but a much lower B_1 value) and the OH-substituted compound **3.1m** (which has a large negative π value). Table 3.5 shows that the K_i and K_i' values that were predicted for both compounds agree with the experimental values. It

Table 3.5 The Inhibition Constants and Physicochemical Parameters of Substituent X of 6-(pX-Phenyl)Pteridin-4-Ones



No.	X	-Log K_i		-LogK_i'		B_1[d]	MR[e]	π[e]
		Exp.[a]	Calc.[b]	Exp.[a]	Calc.[c]			
3.1a	-H	0.770	0.901	0.347	0.365	1.00	1.03	0.00
3.1b	-F	0.699	0.641	0.260	0.182	1.35	0.92	0.14
3.1c	-Cl	-0.279	-0.283	-0.342	-0.436	1.80	6.03	0.71
3.1d	-Br	-0.398	-0.459	-0.653	-0.527	1.95	8.88	0.86
3.1e	-I	-0.580	-0.259	-0.352	-0.417	2.15	13.59	1.12
3.1f	-methyl	-0.380	-0.039	-0.477	-0.262	1.52	5.65	0.56
3.1g	-ethyl	-0.041	0.075	-0.176	-0.393	1.52	10.30	1.02
3.1h	-*n*-propyl	0.284	0.383	-0.380	-0.293	1.52	14.94	1.55
3.1i	-*i*-propyl	0.301	0.081	-0.398	-0.421	1.90	14.94	1.53
3.1j	-*t*-butyl	0.347	0.487	-0.279	-0.265	2.60	19.62	1.98
3.1k	-methoxy	-0.176	0.017	0.075	0.074	1.35	7.87	-0.02
3.1l	-*n*-butyl[f]	0.824	1.361	-0.146	0.068	1.52	19.62	2.08
3.1m	-OH[f]	0.523	0.357	0.347	0.449	1.35	2.85	-0.67

a) Calculated with the values in Table 3.1.
b) Calculated withEquation 3.3.
c) Calculated withEquation 3.7.
d) Reference 16b.
e) Reference 16a.
f) These compounds were not used to develop QSAR Equations 3.3 and 3.7.

is especially remarkable that compound **3.11**, which has such a large substituent, also has such a small K_i value. Nevertheless for a good agreement with the calculated value the measured K_i value should be even smaller.

Finally, a QSAR analysis of all the synthesized derivatives was done. This produced one last equation for the free enzyme:

$$-\log K_i = -\ 0.599\ B_1\ (t = 3.1) + 0.011\ (MR)^2\ (t = 6.2) - 0.199\ MR\ (t = 5.1) + 1.596 \qquad (3.8)$$

$n = 13 \qquad r = 0.93 \qquad s = 0.21 \qquad F = 18.3$

For the immobilized enzyme, this became:

$$-\log K_i' = -\ 0.262\ B_1\ (t = 2.0) - 0.418\ \pi\ (t = 3.2) + 0.005\ (MR)^2\ (t = 4.5) - 0.076\ MR\ (t = 2.7) + 0.645 \qquad (3.9)$$

$n = 13 \qquad r = 0.93 \qquad s = 0.14 \qquad F = 13.4$

Equations 3.8 and 3.9 are similar to Equations 3.3 and 3.7, except for the larger F value. The QSAR analysis showed a decrease in colinearity (from 0.785 to 0.629 between π and B_1, and from 0.785 to 0.657 between B_1 and MR), but the new values are still not ideal. Equations 3.8 and 3.9 support the results of the previous QSAR analyses. The number of compounds increases accordingly from eleven to thirteen, and the number of parameters remains the same as that used in Equations 3.3 and 3.7. Accordingly for Equation 3.9 there are now at least three compounds for every parameter used.

The results of this QSAR analysis should be interpreted with some caution, especially as the range of the log K_i and log K_i' values is not very large. Nevertheless, the QSAR analysis has enabled a fairly accurate prediction of the inhibition of xanthine oxidase by two other 6-arylpteridin-4-ones, namely **3.11** and **3.1m**.

The findings presented in this chapter show that the shape of a substituent in a compound can have a marked effect on the interaction of that compound with an enzyme. This implies that, up to a four-carbon substituent at the **para** position of a 6-arylpteridin-4-one, a rod-shaped substituted compound will inhibit xanthine oxidase more effectively than a spherical substituted compound. Further studies of other 6-arylpteridin-4-

ones, especially those substituted with acetylamines, which would break the still-high colinearity, are needed to support these conclusions.

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17 If compounds **3.1a**, **3.1f**, **3.1k-m** are incubated alone with xanthine oxidase, a very slow change in the UV spectra occurs. For compounds **3.1a** and **3.1m**, the final spectra were similar to the corresponding lumazines.

4. THE OXIDATION OF 8-ARYLHYPOXANTHINES BY FREE AND IMMOBILIZED XANTHINE OXIDASE

4.1 INTRODUCTION

For many years now, a primary method of gaining information about xanthine oxidase has been to study its interaction with various molecules. This University's Department of Organic Chemistry has already reported on the oxidation of various 6 and 7-arylpteridin-4-ones by milk xanthine oxidase [1] and bacterial xanthine oxidase [2]. Another interesting group of compounds is that made up of the **para**-substituted 8-arylhypoxanthines. Bergman et al. [4] have described the oxidation of three of these, namely X = H, methyl, and nitro. Their rate of oxidation is very low compared with that of xanthine. Bergman et al. reported only relative oxidation rates, so it is difficult to calculate the V_{max} and K_m values for a larger series of compounds. Accordingly, part of the work done for this thesis was to obtain more accurate measurements of the 8-arylhypoxanthines.

This chapter describes the effect of various pH levels, temperatures, and organic solvents on the oxidation of the 8-arylhypoxanthines to the corresponding 8-arylxanthines by bovine milk xanthine oxidase.

4.2 MATERIALS AND METHODS

4.2.1 Materials

The bovine milk xanthine oxidase (E.C. 1.2.3.2) came from Boehringer. The CNBr-activated Sepharose 4B came from Pharmacia. The UV spectra and kinetic measurements were made either with a Varian DMS-100 spectrophotometer equipped with a DS-15 data station, or with an LKB Ultrospec II spectrophotometer coupled to an Apple IIe personal computer. The IR spectra were done with a Jasco A-100 infrared spectrophotometer. The mass spectra were done with an AEI MS-902 apparatus.

4.2.2 Enzymatic Oxidation

The xanthine oxidase assay consisted of 2.5 ml of a solution containing 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 90 μg xanthine oxidase, and 2-30 μM of substrate. The oxidation rates were measured at various wavelengths with an LKB Ultrospec II spectrophotometer coupled to an Apple IIe personal computer. Each determination was done in triplicate for at least six different substrate concentrations.

The kinetic parameters were then calculated with the Hanes-Woolf plot [5] on a Varian DS-15 computer station using the Enhanced Kinetics Calculations Program (85-100542-00). The appropriate wavelengths and corresponding mean molar differential-absorption coefficient for the measurements of the oxidation rates of the 8-arylhypoxanthines were determined with a DMS-100 spectrophotometer (Table 4.1).

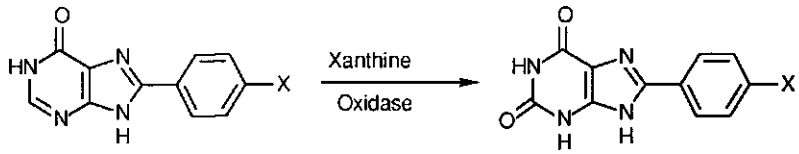
The xanthine oxidase was then immobilized on *n*-octylamine Sepharose 4B according to previously described methods [6]. The immobilized xanthine oxidase was packed in a small column (approximately 1.5 ml) and used to oxidize some 8-arylhypoxanthines. The flowrate was so much optimized that the column was able to convert all the xanthine at the beginning of the experiment.

Table 4.1 Wavelength and Molar-Extinction Coefficient Used for the Enzymatic Assays of Some 8-Arylhypoxanthines

No.	X	pH	Wavelength (nm)	Log δε
4.1a	H	7.5	319	4.061
		8.0	321	4.037
		8.4	325	4.004
		8.8	324	3.995
4.1b	CH_3	7.5	323	3.862
		8.0	323	4.049
		8.4	325	4.079
		8.8	326	4.083
4.1h	Br	7.5	329	3.886
		8.0	330	3.916
		8.4	332	4.064
		8.8	332	4.053

4.3 RESULTS AND DISCUSSIONS

Xanthine oxidase causes a very slow oxidation of the 8-arylhypoxanthines (**4.1**) at position 2, producing 8-arylxanthine (**4.2**). This finding, illustrated in Scheme 4.1, was proved by the ^{1}H-NMR spectra of the isolated product (**4.2a**), which showed that the characteristic single absorption of H-2 at δ = 8.1 ppm is missing.



4.1a-k **4.2a-k**

X		X		X	
a:	H	**e**:	*t*-C_4H_9	**i**:	NO_2
b:	CH_3	**f**:	F	**j**:	$N(CH_3)_2$
c:	*i*-C_3H_7	**g**:	Cl	**k**:	OCH_3
d:	*n*-C_4H_9	**h**:	Br		

Scheme 4.1

Because the reaction rate of xanthine oxidase with the 8-arylhypoxanthines is so slow, only a few of the compounds that had been synthesized earlier (Chapter 2) were suitable for detailed experiments on kinetic parameters. Consequently, this thesis describes the oxidation pattern of only three of the 8-arylhypoxanthines, namely the unsubstituted compound **4.1a** (X = H), the **p**-methyl compound **4.1b** (X = CH_3), and the **p**-bromo compound **4.1h** (X = Br).

Often, a slow oxidation rate means that a compound has a low affinity for the enzyme, but **4.1a**, **4.1b**, and **4.1h** have a low K_m value, which indicates that the binding between xanthine oxidase and the 8-arylhypoxanthines is very tight (Table 4.2).

Table 4.2 Influence of pH on the K_m and V_{max} Values of 8-Arylhypoxanthines at 30°C [a)]

No.	pH = 7.5		pH = 8.0		pH = 8.4		pH = 8.8	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
4.1a	0.99	0.28	1.40	0.60	1.79	0.97	2.45	0.47
4.1b			0.63	0.19	0.96	0.11	1.30	0.26
4.1h			1.17	0.34	1.40	0.26	2.14	0.10
Xanthine	25.9	0.08	44.7	3.22	55.0	8.46	68.1	19.44

a) K_m (µM) and V_{max} (10^{-2} µmole/min/mg protein) were measured using assays as described in Materials and Methods.

Figure 4.1 illustrates the effect of pH on the V_{max} of xanthine oxidase with various substrates. All of the curves show that oxidation activity increases with pH. The K_m values of the 8-arylhypoxanthines, however, do not change as profoundly with pH as do those of xanthine.

Although it would have been interesting to test the oxidation pattern of the 8-arylhypoxanthines at a higher pH for a maximum V_{max} or K_m, the capacity of the phosphate buffer would not have been sufficient under such conditions. Table 4.3 presents the V_{max} and K_m values of 8-phenylhypo-

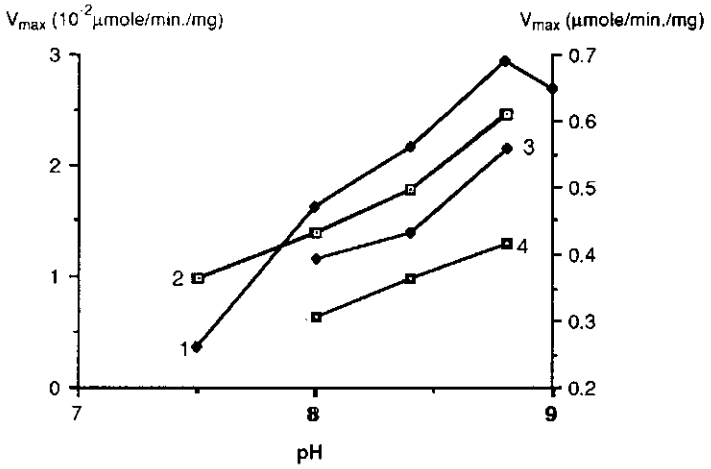


Figure 4.1 Effect of pH on the oxidation rate of xanthine oxidase with various substrates. (Assays were performed as described in Materials and Methods. The substrates used were 1.) xanthine (right Y axis), 2.) 8-phenyl-hypoxanthine, 3.) 8-(**p**Br-phenyl)hypoxanthine, and 4.) 8-(**p**CH_3-phenyl)hypoxanthine.)

xanthine (**4.1a**) in different buffer systems. The oxidation rate of 8-phenylhypoxanthine is considerably lower in Tris-HCl buffer and borate buffer than in phosphate buffer. The K_m value is also lower. This means that, as the buffer solution affects the oxidation of the 8-arylhypoxanthines by xanthine oxidase, it is virtually impossible to compare the V_{max} and K_m values at a higher pH, when the buffer solution is different.

Table 4.3 V_{max} and K_m Values of 8-Phenylhypoxanthine at 30°C in Different Buffer Systems [a)]

Buffer	pH	V_{max}	K_m
Phosphate	6.9	0.50	0.13
	7.5	0.99	0.28
	8.0	1.40	0.60
	8.4	1.79	0.97
Tris/HCl	7.6	0.30	0.10
	8.0	0.30	0.12
	8.5	0.90	0.11
Borate	8.5	0.90	0.18
	9.0	1.10	0.18
	9.5	1.50	0.19

a) K_m (μM) and V_{max} (10^{-2} μmole/min/mg protein) were measured using assays as described in Materials and Methods. The ionic strength of all the buffers was 0.01.

Table 4.4 shows the influence of temperature on the V_{max} and K_m values of the 8-arylhypoxanthines. The V_{max} values increase with temperature. The K_m values also increase, except for the bromo compound (**4.1h**), where K_m is lower at 30°C than at 25°C or 37°C.

Table 4.4 Influence of Temperature on the K_m and V_{max} Values at pH 8.8 [a)]

No.	T = 20°C		T = 25°C		T = 30°C		T = 37°C	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
4.1a	1.03	0.24	1.48	0.18	2.45	0.47	4.27	0.9
4.1b			0.90	0.07	1.30	0.26	2.67	0.31
4.1h			1.51	0.18	2.14	0.10	3.65	0.29

a) K_m (μM) and V_{max} (10^{-2} μmole/min/mg protein) were measured using assays as described in Materials and Methods.

To isolate the enzymatic product of the 8-arylxanthine for further analysis and characterization, a reaction was set up on a small, laboratory scale (approximately 1 g). For these experiments, xanthine oxidase immobilized on *n*-octyl CNBr-activated Sepharose 4B was used. Because they enhance the solubility of substrates, various alcohols and DMSO were also tested for their effectiveness as organic cosolvents.

Only low percentages of organic cosolvent were used, for experiments have shown that the initial oxidation rate decreases considerably if the concentration of the organic cosolvent is more than 10% (Table 4.5).

Table 4.5 Initial Enzymatic Activity of Xanthine Oxidase in the Presence of Organic Cosolvents

Organic Solvent[a)]	Percentage (v/v)						
	0%	5%	10%	15%	20%	30%	50%
Ethanol	100%	89%	67%	46%	27%	18%	<5%
2-Butoxyethanol	100%	88%	59%		53%	44%	40%

a)The water part of the solvent system contained 50 mM phosphate buffer (pH 8.1) and 50 µM EDTA. The xanthine concentration was 65 µM.

Except for 10% 2-butoxyethanol and 20% *n*-propanol, organic cosolvents do not slow the oxidation rate during long reactions (Table 4.6). Interestingly enough, with 10% *n*-propanol and 10% DMSO, immobilized xanthine oxidase has even more operational stability, proof that 10% DMSO is an effective organic cosolvent in the enzymatic oxidation of the 8-arylhypoxanthines.

The substrates were dissolved in 25 mM phosphate buffer (pH 8.1) containing 50 µM EDTA and 10% DMSO at a concentration of 10 mM (approximately 2 gr/ltr). At a flow rate of 15 ml/hour, a complete oxidation of compounds **4.1a**, **4.1b**, and **4.1h** was possible for approximately 20 hours. Based on the UV spectra of the product stream, these conversion percentages mean that about 600 mg of the compounds were ultimately oxidized into the corresponding 8-arylxanthines (**4.2**).

Additional evidence of 8-arylxanthine formation was obtained from the isolation and subsequent structure identification of compound **4.2b** by ^{1}H-NMR and IR spectra. The ^{1}H-NMR spectrum of this compound was missing a singlet absorption of H-2 around 8 ppm. The IR spectrum showed a double

Table 4.6 Half-Lives of Immobilized Xanthine Oxidase with Different Organic Cosolvent During Continuous Catalysis

Solvent System[a]	Time[b] (100% conversion)	Half-Life [c]
100% water	10 hours	13.5 hours
10% ethanol	12 hours	23 hours
10% *n*-propanol	18 hours	27 hours
10% *t*-butanol	11 hours	13.5 hours
10% 2-butoxyethanol	6 hours	8 hours
10% dimethylformamide	10 hours	15 hours
10% dimethylsulfoxide	29 hours	43 hours
20% *n*-propanol	2 hours	5 hours

a)The water part of the solvent system contained 50 mM phosphate buffer (pH 8.1) and 50 μM EDTA. The xanthine concentration was 65 μM. The flow was so much optimized that the column was able to convert all the xanthine at the beginning of the stability test.

b)Time at which the column starts to convert less then 100% of the xanthine.

c)Time at which the column starts to convert only 50% of the xanthine.

absorption peak around 1700 cm^{-1} and an additional broad band at 3500 cm^{-1}. It is especially this double absorption at 1700 cm^{-1} that indicates the presence of two C = O groups in the molecule. The IR spectrum of compound **4.2a** showed a broad-band absorption at 3500 cm^{-1} and a broad-band absorption at 1700 cm^{-1}. In contrast, the starting compound (**4.1a**) had only a narrow absorption peak at 1700 cm^{-1}.

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5 QSAR STUDIES OF THE INHIBITION OF FREE XANTHINE OXIDASE BY 8-ARYLHYPOXANTHINES

5.1 INTRODUCTION

The synthesis of the 8-arylhypoxanthines (**5.1a-k**) was described in Chapter 2 of this thesis. Their oxidation into 8-arylxanthines (**5.2a-k**) by xanthine oxidase was described in Chapter 4. Unfortunately, only a few of these compounds were suitable for more detailed studies of oxidation by xanthine oxidase. This was because the 8-arylhypoxanthines had very low reaction rates, which made it difficult to measure accurately the enzymatic parameters K_m and V_{max} and examine the influence of the substituents (X) on those parameters.

The very low K_m values obtained during oxidation indicate a tight binding between the 8-arylhypoxanthines and xanthine oxidase [1]. Nevertheless, the V_{max} and K_m values do not give sufficient quantitative information about the binding and interaction between the 8-arylhypoxanthines and xanthine oxidase. Accordingly, the initial purpose of the experiments described here was to assess how well the 8-arylhypoxanthines inhibit the ability of xanthine oxidase to convert xanthine to uric acid.

The V_{max} of the 8-arylhypoxanthines is so low that, in the time it takes to inhibit the oxidation of xanthine, one would expect only a negligible amount of the 8-arylhypoxanthines to have been oxidized. Because of their strong affinity for xanthine oxidase, it is assumed that the 8-arylhypoxanthines can compete successfully with xanthine for the active sites of the enzyme, thus inhibiting the oxidation of the substrate (xanthine) upon binding. The resulting inhibition constants would then reflect the effect of the substituents (X) at the **para** position of the aryl group on the interaction between the 8-arylhypoxanthines and xanthine oxidase.

Although the literature contains a few isolated reports of 8-arylhypoxanthines with a remarkably low I_{50} value for xanthine oxidase [2], no comprehensive study of them has ever been made.

The inhibition constants for the 8-arylhypoxanthines supplement previous research [3] on the inhibitory properties of the 6-**p**-X-arylpteridin-4-ones (**5.3**). Chapter 3 describes how it was possible to calculate a

quantitative structure-activity relationship (QSAR) for the **para** substituents of these compounds and thus accurately predict that rod-shaped alkyl substituents like *n*-butyl would be more tightly bound to inhibitors. The basic structure of the 8-arylhypoxanthines has two features in common with that of the 6-arylpteridin-4-ones. These are:

- A heterocyclic system with a pyrimidine ring and a hydroxy group at position 4;
- A pyrimidine ring fused at the 5,6-bond with another heterocyclic ring system (pyrazine in the 8-arylhypoxanthines or an imidazole in the 6-arylpteridin-4-ones).

The oxidation pattern of both compounds leads one to assume that they will be fixed to the enzyme at the pyrimidine part [4]. Other studies [5] seem to indicate that the aryl group is instrumental in a tight enzyme-inhibitor complex. The main difference between the two compounds is that, in the 8-arylhypoxanthines, the aryl group aligns with the heterocyclic ring system (parallel to the X axis) and, in the pteridine system, it forms an angle with the heterocyclic ring system (Figure 5.1).

Consequently, the inhibition experiments done for this thesis had a twofold purpose; they would establish whether the structural difference between the 6-arylpteridin-4-ones and the 8-arylhypoxanthines affects the affinity of xanthine oxidase for these two compounds and they would shed more light on the interaction between the aryl group and the hydrophobic area in the enzyme.

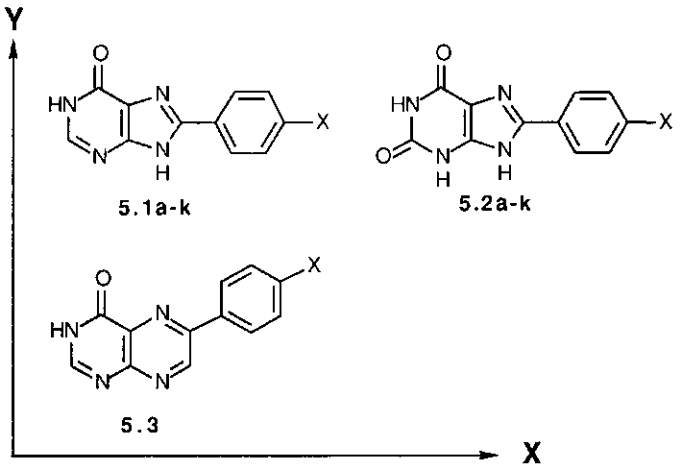


Figure 5.1 Orientation of the aryl-substituent in the 6-arylpteridin-4-ones and the 8-arylhypoxanthines.

5.2 MATERIALS AND METHODS

5.2.1 Materials

The milk xanthine oxidase was purified out of raw, unpasteurized milk from this University's farm according to the method described by Massey et al. [6]. The enzyme was purified to a specific activity of 1.42 units/mg protein. The AFR value was 65 [7]. The xanthine came from Fluka. The allopurinol came from Sigma. All the other chemicals were analytical grade from Merck.

5.2.2 Enzyme Assays

The rates were measured with a Beckman DU-7 spectrophotometer equipped with a Kinetics module and a thermostatted cell holder (25°C). The inhibition of the conversion of xanthine into uric acid was measured at 296 nm ($\delta\varepsilon$ = 10.19 $mM.cm^{-1}$). The assays were performed in 0.1 M sodium phosphate buffer (pH 7.5, 0.1 mM EDTA), consisting of 60 µM xanthine, 20 µg/ml xanthine oxidase, and appropriate amounts of inhibitor in a final volume of 2.5 ml. The I_{50} values (i.e. the concentration at which a compound shows an inhibition of 50%) were calculated by linear regression of the reaction rate versus the log value of the inhibitor concentration.

5.3 RESULTS

5.3.1 Inhibition of Xanthine Oxidase

The initial inhibition experiments tested the effect of pH levels on the enzymatic oxidation of the 8-arylhypoxanthines into 8-arylxanthines. The results showed that the rate of oxidation slows as the pH decreases (Figure 5.2), dropping to almost zero under nearly neutral or acid conditions. As the oxidation rate also slows at lower temperatures [8], subsequent

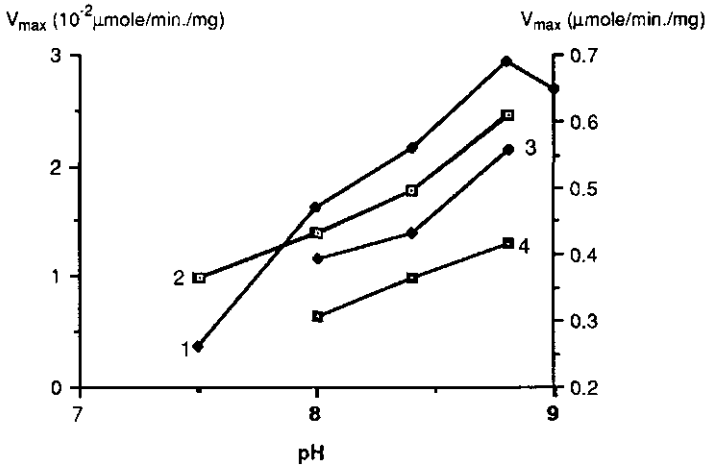


Figure 5.2 Effect of the pH on the oxidation rate of xanthine oxidase with various substrates. Assays were performed as described in Materials and Methods. The substrates used were 1.) xanthine (right Y axis), 2.) 8-phenylhypoxanthine, 3.) 8-(**p**Br-phenyl)hypoxanthine, and 4.) 8-(**p**CH_3-phenyl)hypoxanthine.

experiments were performed at a pH of 7.5 and a temperature of 25°C [9].

The inhibition of xanthine oxidase begins at very low concentrations of the 8-arylhypoxanthines. In fact, a significant decrease in oxidation occurs at equimolar concentrations of the enzyme and inhibitor, which is a clear indication that the inhibitor is very tightly bound. Some 8-arylhypoxanthines, namely X = F, Br, and *t*-butyl, reduced the enzymatic activity to as little as 10% at inhibitor concentrations only ten times higher than the enzyme concentration.

At such low concentrations of the inhibitor, the Lineweaver-Burk equation is not suitable for calculating the kinetic parameters. This equation, which is based on Michaelis-Menten kinetics, assumes that there is no depletion of the inhibitor by the enzyme during oxidation. In other words, it does not allow for the possibility that the binding of the inhibitor to the enzyme can significantly change the concentration of the free inhibitor.

The results of the experiments were even more difficult to analyze because of the non-linear initial rates of the oxidation of xanthine in the presence of an inhibitor, which made it impossible to use the Dixon plot for tightly-bound inhibitors [10]. This problem was circumvented by expressing inhibition as I_{50} values (i.e. the concentration of the inhibitor at which the enzymatic oxidation of xanthine is reduced by 50%). The I_{50} values of the 8-arylhypoxanthines (Table 5.1) were calculated by measuring the initial oxidation rate of 60 μM xanthine and 20 μg/ml protein in at least twelve different concentrations of the inhibitor.

Table 5.1 I_{50} and I_{90} Values of the 8-Arylhypoxanthines and 8-Arylxanthines [a]

		8-Arylhypoxanthine	8-Arylxanthine	
No.	X	I_{50} (μM)	I_{50} (μM) [b]	I_{90} (μM) [b]
5.1a	H	0.21	1.91	27.68
5.1b	F	0.04	1.87	25.04
5.1c	Cl	0.06	1.24	16.49
5.1d	Br	0.01	0.55	12.63
5.1e	CH_3	0.07	2.12	14.43
5.1f	*i*-C_3H_7	0.17	1.27	13.20
5.1g	*n*-C_4H_9	0.09	0.75	88.70
5.1h	*t*-C_4H_9	0.03	2.14	20.90
5.1i	NO_2	0.16	4.22	43.60
5.1j	$N(CH_3)_2$	0.10	1.49	26.80
5.1k	OCH_3	0.11	3.12	30.50
	Allopurinol	4.91	13.21	
	6-Phenylpteridin-4-one	1.70		

a) Assays were performed with 60 μM xanthine and 20 μg/ml xanthine oxidase (AFR = 65 [7]).

b) These I_{50} and I_{90} values were measured after preincubating the inhibitor with xanthine oxidase for an appropriate time (at least one hour for the lower concentrations), to ensure that the inhibitor was oxidized to the corresponding 8-arylxanthine.

The experiments revealed that a sudden increase occurs in the rate of the oxidation of xanthine by the 8-arylhypoxanthines (Figure 5.3). The time that elapses between the start of the reaction and this increase (indicated by the arrows) depends on the concentration of the inhibitor. One can assume then that, at the moment of increase, all of the inhibitor will have been oxidized to the corresponding 8-arylxanthines (**5.2a-k**) and that these products are a less effective inhibitor of xanthine oxidase than the 8-arylhypoxanthines.

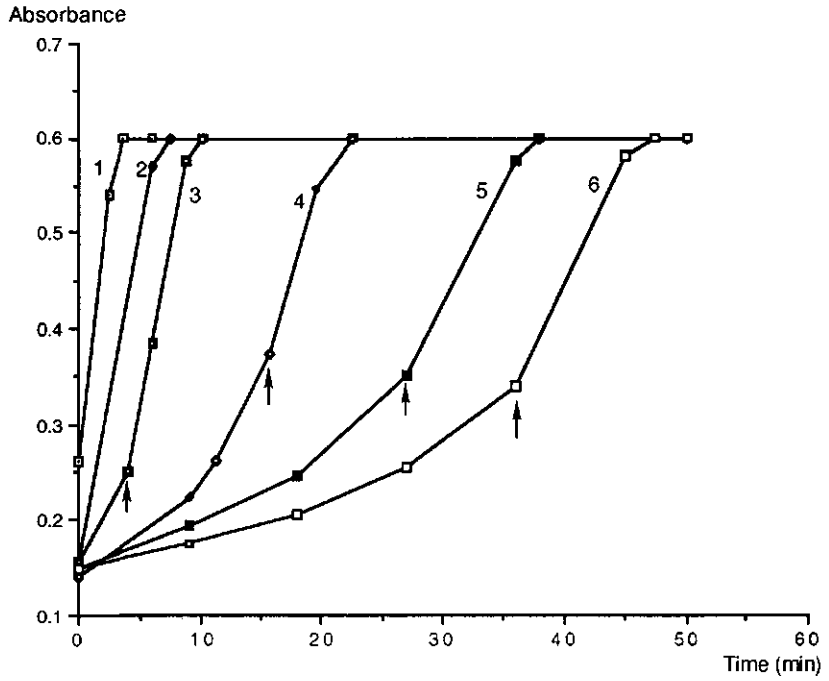


Figure 5.3 Time scan of the inhibited oxidation of xanthine by 8-(pN$(CH_3)_2$-phenyl)hypoxanthine. The xanthine oxidase assays were performed as described in the section on materials and methods. The inhibitor concentrations used were 1.) 0 μM, and 0.078 μM, 2.) 0.156 μM, 3.) 0.391 μM, 4.) 0.703 μM, 5.) 1.016 μM, and 6.) 1.641 μM. The arrows indicate the moment at which a sharp increase in the oxidation rate occurs.

Some of the experiments on inhibition involved testing the 8-arylxanthines. In these experiments, the enzyme was preincubated with the 8-arylhypoxanthines in the phosphate buffer for one to six hours, during which time the 8-arylhypoxanthines were converted fully into the 8-arylxanthines. Subsequently, the inhibitory activity of the 8-arylxanthines was measured, with xanthine as the substrate. The results (Table 5.1) indicate that the I_{50} values of the 8-arylxanthines are ten to fifty times greater than those of the 8-arylhypoxanthines, but still considerably smaller than those of allopurinol or the 6-arylpteridin-4-ones [3].

It is remarkable that preincubation of the enzyme in a low concentration of compound **5.1a-k** (< 150 nM) causes no significant decrease in the enzymatic oxidation rate of xanthine and that preincubation without compound **5.1a-k** causes a decrease of around 5%. This means that low concentrations of the 8-arylhypoxanthines do not affect the activity of xanthine oxidase.

5.3.2 QSAR Analysis

The I_{50} values for the 8-arylhypoxanthines vary greatly. To form a clear picture of the effect of the substituents on the aryl group, these values were used to calculate the QSAR of compounds **5.1a-k** and **5.2a-k**. Table 5.2 shows the results of these calculations for compound **5.1**.

Table 5.2 QSAR Calculation for the Inhibition of Xanthine Oxidase by Substituted 8-Arylhypoxanthines

$I_{50} = -0.105\ B_1 + 0.256\ L - 0.038\ \sigma - 0.180$				(5.1)
(t = 2.0)	(t = 1.2)	(t = 0.7)		
n = 11	r = 0.606	s = 0.065	F = 1.36	
$I_{50} = 0.114\ MTD^{*} + 0.073$				(5.2)
(t = 5.2)				
n = 11	r = 0.866	s = 0.036	F = 26.96	

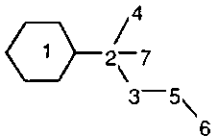
Equation 5.1 gave the best possible results. It is based on the Hansch parameters and the steric parameters of the Sterimol method (Chapter 1). The intercorrelation of these parameters is shown in Table 5.3. The number of parameters was limited to three because only eleven compounds were available. Equation 5.1 has no statistical significance because r and F are very low.

Table 5.3 Squared-Correlation Matrix Showing the Degree of Colinearity (r^2) Between the Variables Used

	π	σ	L	B_1	$(MR)^2$
π	1.000	0.287	0.729	0.659	0.843
σ		1.000	0.175	0.140	0.440
L			1.000	0.362	0.778
B_1				1.000	0.525
$(MR)^2$					1.000

Equation 5.2 is based on the MTD* method (Chapter 1). Although the MTD* parameters seem to explain all the I_{50} values, they give no predictive values outside the congeneric group.

The steric requirements for a good inhibitor are listed in Figure 5.4. They give an idea of other substituents only as long as these substituents fit into the hypermolecule. One example is the 8-(**p**CN-phenyl)hypoxanthine, which has an MTD* value of zero. Unfortunately an attempt to synthesize this compound was unsuccessful (Chapter 4).



1, 2, 7 Are favorable
4, 5, 6 Are indifferent
3 Is unfavorable

Structure	Occupied Position	MTD*
phenyl	1	2
pF-; **p**Cl-; **p**Br-phenyl	1 2	1
pCH_3-phenyl	1 2	1
pOCH_3-phenyl	1 2 3	2
p*i*Pr-; **p**NO_2-phenyl	1 2 3 4	2
p$N(CH_3)_2$-phenyl	1 2 3 4	2
p*n*C_4H_9-phenyl	1 2 3 5 6	2
p*t*C_4H_9-phenyl	1 2 3 4 7	1

Figure 5.4 Hypermolecule for all the positions of the 8-arylhypoxanthines.

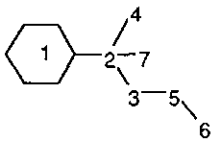
When Equation 5.2 is applied to the 8-arylhypoxanthines, the results show that the linear alkyl substituents do not bind well with xanthine oxidase. This is contrary to the results for the 6-arylpteridin-4-ones (**5.3**), which showed that the *n*-butyl substituents had a very low K_i value (Chapter 3). The I_{50} values of the 8-arylhypoxanthines in Table 5.1 are approximately ten to a hundred times lower than those of the 6-arylpteridin-4-ones. For a view of the real biological activity of the 8-arylhypoxanthines, one would also have to study the inhibitory properties of the oxidized 8-arylhypoxanthines.

Table 5.1 lists the I_{50} and I_{90} values for the 8-arylxanthines. The I_{90} values were included because it was not possible to calculate a significant QSAR equation for the I_{50} values. The QSAR equations for the I_{90} values are shown in Table 5.4. Equation 5.3 uses two Hansch parameters, namely MR and σ, where σ has a large positive coefficient, and the Sterimol parameter B_1, which has a large negative coefficient. With this combination of coefficients, "bulky" substituents with a small σ value will stimulate the formation of an enzyme-inhibitor complex. Equation 5.4, which is based on the MTD* approach, makes roughly the same prediction(Table 5.4).

Table 5.4 QSAR Calculation for the Inhibition of Xanthine Oxidase by Substituted 8-Arylxanthines

$I_{90} = 0.200\ MR - 53.288\ B_1 + 45.564\ \sigma + 90.768$				(5.3)
(t = 6.3)	(t = 5.4)	(t = 4.2)		
n = 11	r = 0.926	s = 6.15	F = 14.22	
$I_{90} = -8.663\ \pi + 39.154\ MTD^* - 0.073$				(5.4)
(t = 5.2)	(t = 8.1)			
n = 11	r = 0.948	s = 7.77	F = 35.33	

The steric requirements (Figure 5.5) show that xanthine oxidase tolerates occupancy of positions 2, 3, 4, and 7 and that it allows binding to the 8-arylxanthines more readily if the substituents have a hydrophobic property.



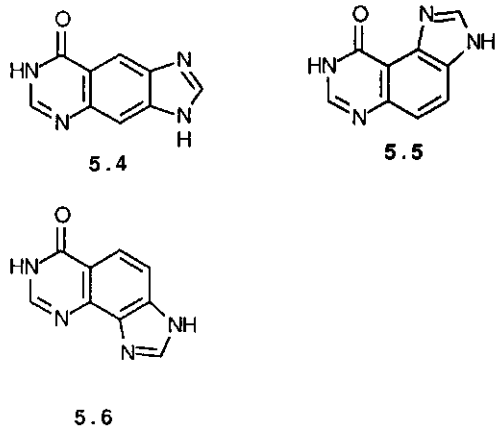
2, 3, 4, 7 Are indifferent
1, 5, 6 Are unfavorable

Structure	Occupied Position	MTD*
phenyl	1	1
pF-; **p**Cl-; **p**Br-phenyl	1 2	1
pCH_3-phenyl	1 2	1
pOCH_3-phenyl	1 2 3	1
p*i*Pr-; **p**NO_2-phenyl	1 2 3 4	1
p$N(CH_3)_2$-phenyl	1 2 3 4	1
p*n*C_4H_9-phenyl	1 2 3 5 6	3
p*t*C_4H_9-phenyl	1 2 3 4 7	1

Figure 5.5 Hypermolecule for all the positions of the 8-arylxanthine.

5.4 DISCUSSION

The inhibitory performance of the 8-arylhypoxanthines and the 8-arylxanthines shows that their affinity for xanthine oxidase is much greater than that of the 6-arylpteridin-4-ones and allopurinol. As stated above, the main steric difference between the 8-arylhypoxanthines and the 6-arylpteridin-4-ones is the position of the aryl group in relation to the heterocyclic system. The linear molecules, namely the 8-aryl(hypo-)xanthines (**5.1a-k** and **5.2a-k**), show a greater affinity for xanthine oxidase than the non-linear molecules, namely the 6-arylpteridin-4-ones (**5.3**). Leonard et al. [11] observed a similar phenomenon when they tested "stretched-out" analogs of hypoxanthine as substrates of xanthine oxidase (Figure 5.6). The linear molecule *lin*-benzohypoxanthine (**5.4**) reacted faster than the angular molecule *prox*-benzohypoxanthine (**5.5**). Leonard et al. also observed that the other angular molecule, *dist*-benzohypoxanthine (**5.6**), reacts just as fast as the *lin*-benzohypoxanthine. The 7-arylpteridin-4-ones have a geometrical conformation similar to that of the *dist*-benzohypoxanthine, but show no inhibitory properties, for they are well oxidized by xanthine oxidase. The K_m value of the *dist*-benzohypoxanthine is of the same order as the K_i value of the 6-arylpteridin-4-ones [3].



5.4 5.5

5.6

Figure 5.6 Stretched-out analogs of hypoxanthine.

A detailed discussion of the effect of the substituents is not possible because both the availability of the required compounds and the predictive capacity of QSAR Equations 5.2 and 5.4 are limited. In general, however, the substituents of the 8-arylhypoxanthines that have an affinity for xanthine oxidase are those that are linear in relation to the X axis of the molecule (Figure 5.1). The 8-arylxanthines, that have an affinity for xanthine oxidase are those with a fairly short substituent. This affinity is enhanced by the hydrophobic character of the substituents.

5.5 REFERENCES

1 See Table 4.2.

2 a) B.R. Baker, W.F. Wood, and J.A. Kozma (1968). *J. Med. Chem.*, **11**, p. 661;

b) F. Bergman, L. Levene, and H. Govrin (1977). *Biochim. Biophys. Acta*, **484**, p. 275.

3 H.S.D. Naeff, H.C. van der Plas, J. Tramper, and F. Müller (1985). *Quant. Struct. Act. Relat.*, **4**, p. 161.

4 R.K. Robins, G.R. Revankar, D.E. O'Brien, R.H. Springer, T. Novinson, A. Albert, K. Senger, J.P. Miller, and D.G. Streeter (1985). *J. Het. Chem.*, **22**, p. 601.

5 B.R. Baker and W.F. Wood (1967). *J. Med. Chem.*, **10**, p. 1101.

6 V. Massey, P.E. Brumby, and H. Komai (1969). *J. Biol. Chem.*, **244**, p. 1682.

7 AFR (Activity-to-Flavin Ratio) is a measure of functional enzyme activity, with AFR = 212 corresponding to a fully active enzyme. AFR is defined as the change in absorbance per minute at 296 nm in a 3 ml reaction volume, divided by the absorbance at 450 nm of the xanthine oxidase used in the assay, under the standard conditions of 100 μM xanthine in 0.1 M pyrophosphate, at pH 8.5 and 25°C. See R.C. Bray (1975). In: P.D. Boyer (editor) *The Enzymes*, **12**, p. 299-419.

8 See Table 4.4.

9 Unlike the experiments described in Chapter 3, where enzymatic inhibition by the 6-arylpteridin-4-ones was measured at pH 6.9, the experiments described here had to be done at a higher pH, as, at pH 6.9, some of the 8-arylhypoxanthines crystallized during the experiments.

10 a) H. Segel (1975). *Enzyme Kinetics*. John Wiley, New York;

b) M. Dixon (1972). *Biochem. J.*, **129**, p. 197.

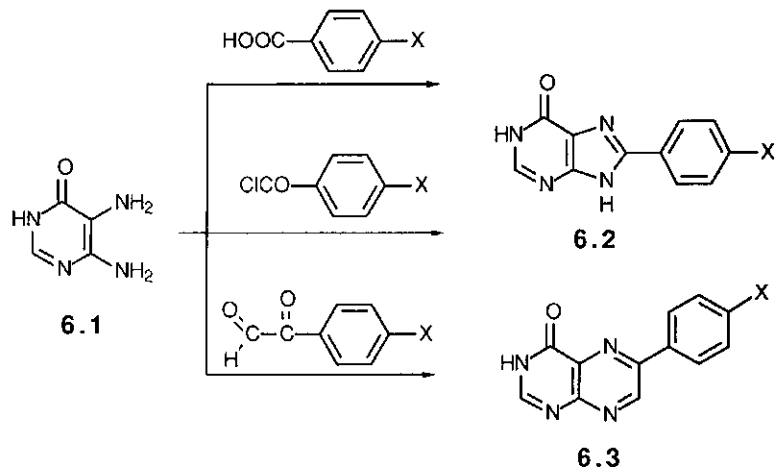
11 N.J. Leonard, M.A. Sprecker, and A.G. Morrice (1976). *J. Am. Chem. Soc.*, **98**, p. 3987.

6. GENERAL DISCUSSION

This thesis presents the results of experiments on the synthesis of the 6-arylpteridin-4-ones and the 8-arylhypoxanthines, an analysis of how well these compounds inhibit the active site of bovine milk xanthine oxidase, and a description of how a quantitative structure-activity relationship (QSAR) was developed to explain their inhibitory activity further. This chapter contains a review of the chemical synthesis of the 6-arylpteridin-4-ones and the 8-arylhypoxanthines, followed by an analysis of their inhibitory properties.

6.1 CHEMICAL SYNTHESIS

For this thesis, simplicity was a primary consideration in the chemical synthesis of the 8-arylhypoxanthines and the 6-arylpteridin-4-ones. This was to facilitate possible future use of the compounds. A feature common to both compounds is the pyrimidine ring, which means that, for the synthesis, one can use the same starting material, namely 4,5-diamino-pyrimidin-6-one (**6.1**).



Scheme 6.1 Synthetic pathways of 8-arylhypoxanthines and 6-arylpteridin-4-ones.

To synthesize the 8-arylhypoxanthines, a condensation of 4,5-diamino-pyrimidin-6-one (**6.1**) with a substituted benzoic acid was sufficient to obtain the required 8-arylhypoxanthines (**6.2**). The dehydration for this step requires polyphosphoric acid, which works conveniently with several substituted benzoic acids. In an attempt to extend the series of 8-arylhypoxanthines with other substituents, substituted benzoylchlorides were used instead of substituted benzoic acid. Although the formation of the intermediate 4-amino-5-arylamidopyrimidin-6-one was attained for several substituted aryl groups, the subsequent ring closure of various 4-amino-5-arylamidopyrimidin-6-ones into the required hypoxanthines was troublesome. In some of the unsuccessful experiments, 4-amino-5-arylamidopyrimidin-6-one was recovered and, in others, compound **6.1** and arylcarboxylic acid were found in the reaction mixture.

The 6-arylpteridin-4-ones (**6.3**) were conveniently synthesized by reacting compound **6.1** with an arylglyoxal in 96% ethanol at pH 2.7 (Chapter 2). The main drawback of this method is that, besides compound **6.3**, one always obtains the isomeric 7-arylpteridin-4-one. A suitable way to separate the two isomers is by recrystallization from DMSO. De Meester et al. [1] developed an unequivocal synthesis of the 6-arylpteridin-4-ones, in which they used appropiately substituted pyrazine N-oxides as starting materials [2]. Difficulties in synthesizing some of the N-oxides, however, made it impossible to obtain more than a very few substituted 6-arylpteridin-4-ones.

6.2 INHIBITORY PROPERTIES

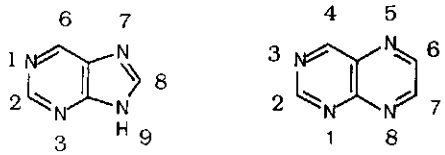
Xanthine oxidase has been intensively studied [3]. It consists of two identical subunits [3a], each of which has a molybdenum-pteridin, an FAD (Flavine Adenine Dinucleotide), and two iron-sulfur centers as cofactors. Xanthine oxidase is interesting to synthetic chemists because it catalyzes the hydroxylation of a wide variety of purines and pteridines. The mechanistic questions concerning the enzymatic reactions have been the subject of many biochemical studies.

Notwithstanding the work of biochemists, the picture of the reaction of xanthine oxidase with reducing substrates is still incomplete. The reduction of xanthine oxidase by xanthine occurs by electron transfer [4].

The currently accepted number of electrons taken up by a fully reduced enzyme is six per subunit: two to molybdenum, two to flavin, and one to each iron-sulfur center. Thus it would require three molecules of substrates reacting sequentially to generate the six-electron reduced enzyme [5].

At which sites, then, is there a possible interaction of substrates (either reducing or oxidizing) with the three prosthetic groups of xanthine oxidase? Olson et al. [6] concluded that the iron-sulfur centers act as electron sinks during turnover to keep molybdenum oxidized and flavin reduced. Bray [3b] supports these findings and even suggests that the iron-sulfur centers may be buried within the molecule. This means that only the molybdenum and the flavin cofactors are available for interaction with substrates. Other investigators [7] have argued that all the prosthetic groups can interact with substrates.

These controversies reflect very well the state of the research on xanthine oxidase. There seems to be a general agreement on the action mechanism, but the reaction mechanism has yet to be described to everyone's satisfaction. It is in this context that compounds **6.2** and **6.3** were used to increase knowledge of the enzyme. Because both sets of compounds are slowly oxidized by xanthine oxidase at the C-2 position (Figure 6.1), the assumption is that these compounds interact at the molybdenum site (Chapters 3 and 4).



6.4 6.5

Figure 6.1 Numbering of the purine and pteridine ring systems.

Robins et al. [8] recently proposed a model of the interaction of molybdenum with organic compounds. This model includes the pterin cofactor with molybdenum to propose a kinetic model of the oxidation of hypoxanthine and xanthine (Figures 6.2 and 6.3). The pterin cofactor brings the enzymatic nucleophilic site and the molybdenum atom in the active pocket together in the right orientation for the oxidative reaction. The model has two types of substrate binding: type 1 and type 2. Figure 6.2

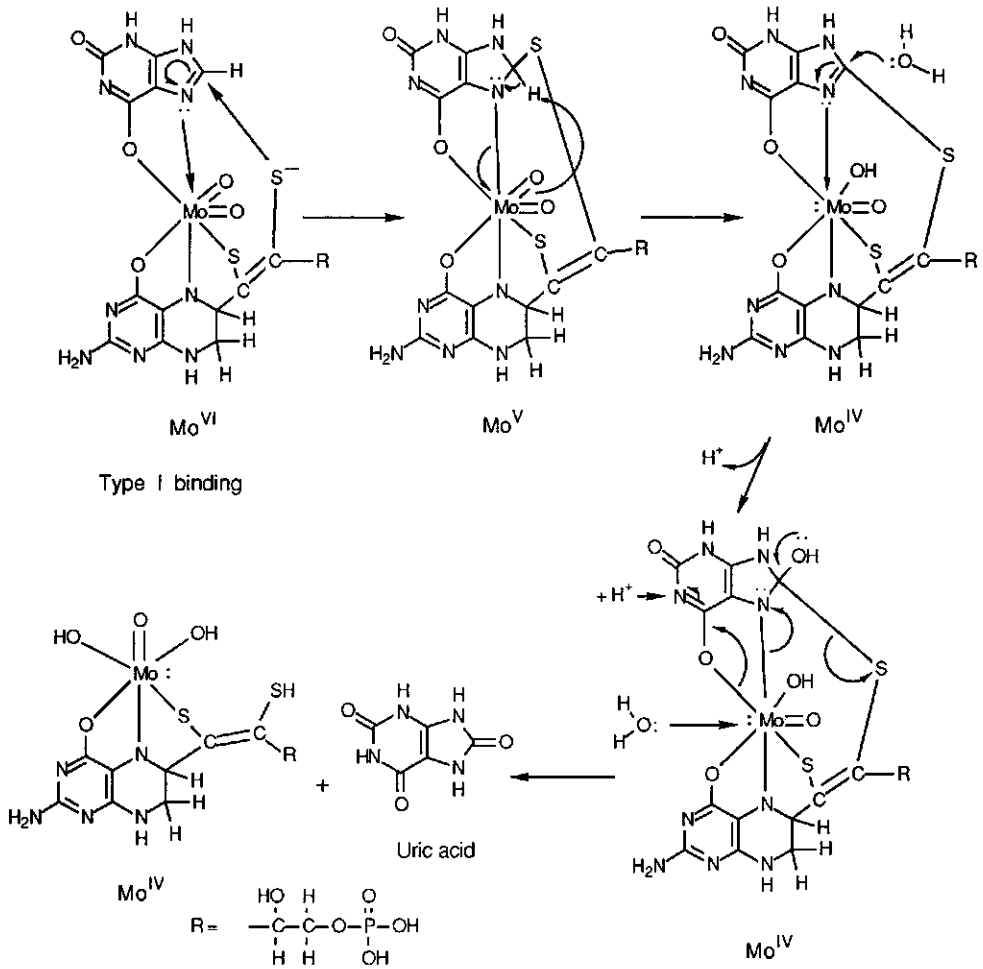


Figure 6.2 Binding pattern of xanthine with molybdopterin and oxidation of xanthine into uric acid by xanthine oxidase. (After Robins et al. [8].)

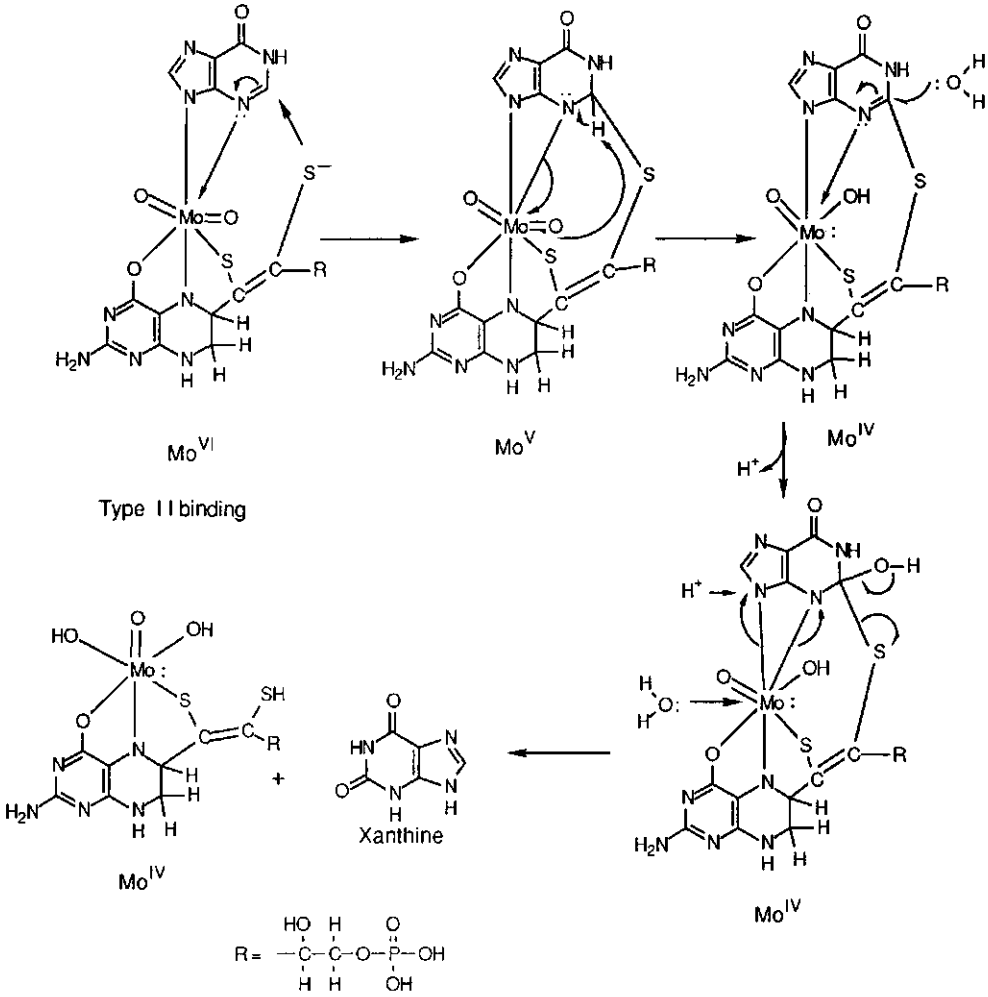


Figure 6.3 Binding pattern of hypoxanthine with molybdopterin and oxidation of hypoxanthine into xanthine by xanthine oxidase. (After Robins et al. [8].)

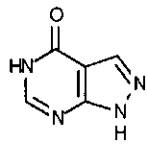
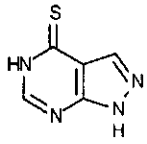
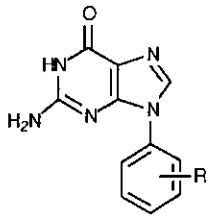
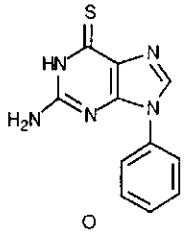
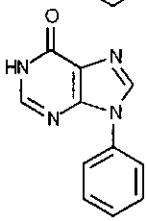
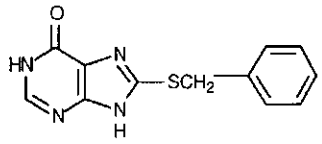
shows how xanthine binds with the molybdopterin cofactor at O-6 and N-7 in a type I binding. Figure 6.3 shows how hypoxanthine binds with the molybdopterin cofactor at N-3 and N-9 in a type II binding. Either way, the sulfide group is oriented properly for the attack on C-8 of xanthine or C-2 of hypoxanthine. By varying the substitution pattern of the substrates or the inhibitors, one can vary the dimensions and explore the active site.

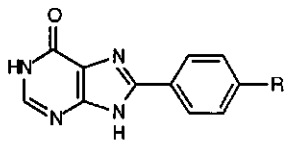
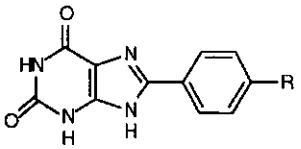
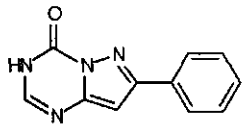
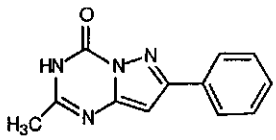
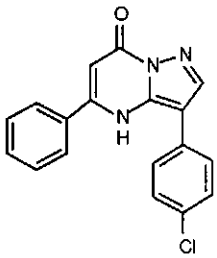
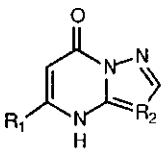
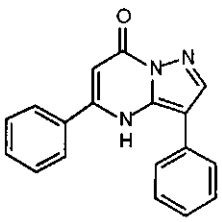
The recent synthesis of benzo-elongated purines [9] and pteridines [10], and their subsequent oxidation by xanthine oxidase, shows that the active site can bind large compounds in a reactive binding mode. Moder and Leonard [11] found the spatial limit for the reactive type I binding region by synthesizing *lin*-naphthoxanthine (**6.u**, Table 6.1), which is not oxidized by xanthine oxidase. The type II binding region seems to be larger, for *lin*-naphthohypoxanthine (**6.8**, Table 6.2) is still oxidized to compound **6.u** [11].

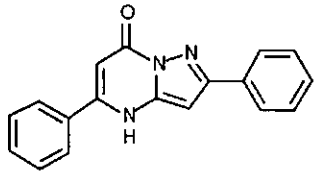
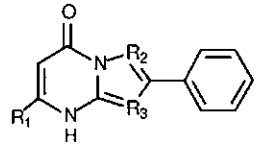
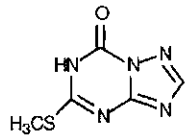
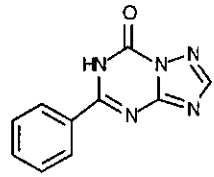
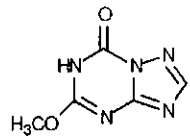
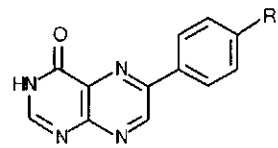
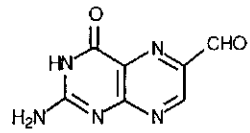
As to the binding of inhibitors by xanthine oxidase, the variation in size and structure is almost limitless. Table 6.1 gives some examples of the compounds used in the inhibition studies.

Most of the inhibitors are modeled on a purine-type azaheterocyclic ring system. If one follows the numbering system of purine (**6.4**), the nitrogen atom at position 1 seems essential to the good inhibitory properties of a compound, for compounds **6.j-n** have large I_{50} values. At the same time, an aryl substituent connected to the five-membered ring enhances the binding with the enzyme considerably, for compounds **6.c-h** have smaller I_{50} values than compounds **6.a** and **6.b**. The active site is flexible with inhibitors, for it accommodates small compounds (**6.a** and **6.b**) very well. On the other hand, it also accommodates compounds like **6.j**, **6.l**, and **6.m**, which have two aryl substituents, and the elongated *lin*-naphthoxanthine (**6.u**). The inhibitor-enzyme complex for the larger compounds is not as strong as that for the smaller compounds because the I_{50} and K_i values are larger. Nevertheless, the enzyme is still able to accommodate that type of inhibitor in the active site. Mono-aryl compounds with a type II binding seem to bind better with the enzyme than those with a type I binding (compare, for example, compounds **6.2** and **6.f**, which have a type II binding, with **6.e**, which has a type I binding, or compare compound **6.h** with **6.i**). One explanation of this could be that the aryl substituent in a type II binding has a better interaction with the hydrophobic "wall" of the active site than the aryl substituent in a type I binding.

Table 6.1 Hypoxanthine, Xanthine, and Pteridine Analogs as Inhibitors of Xanthine Oxidase

No.	Compound	I_{50} (μM)	Ref.
6.a		4.9	12
6.b		8.4	12
6.c		R = H 0.41 R = pCl 1.8	13
6.d		1.1	13
6.e		13	14
6.f		2.8	15

No.	Compound	I_{50} (μM)	Ref.
6.2		R = H 0.209 R = Br 0.010	16
6.g		R = H 1.9 R = Br 0.55	16
6.h		0.047	17
6.i		80	18
6.j		40	19
6.k		R_1= H R_2= CH 11 R_1= H R_2= N 45 R_1= Cl R_2= CH 24 R_1= Cl R_2= N 78 R_1= OH R_2= N >150	20
6.l		91	19

No.	Compound	I_{50} (μM)	Ref.
6.m		160	19
6.n		$R_1 = CH_3$; $R_2 = CH$; $R_3 = N$ >120 $R_1 = H$; $R_2 = N$; $R_3 = CH$ 50	21
6.o		1.4	8
6.p		21	8
6.q		80	8
6.3		R = H 0.17 R = nC_4H_9 0.15	22
6.r		$K_i = 6 \times 10^{-4}$ μM	23

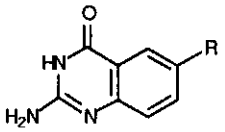
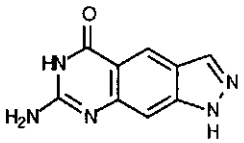
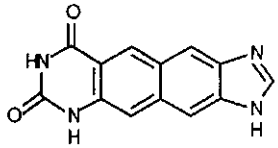
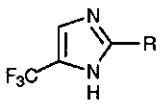
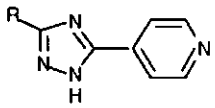
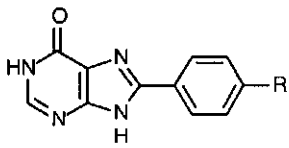
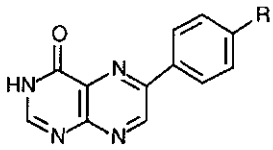
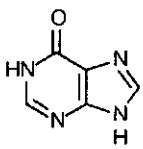
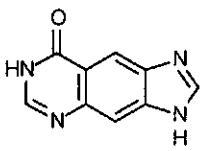
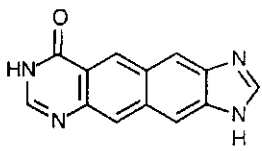
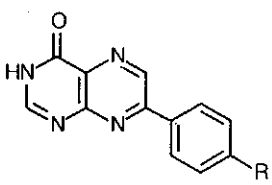
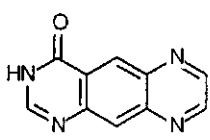
No.	Compound	I_{50} (μM)	Ref.
6.s		R = H 0.9 R = CH_3 1.2 R = CHO 0.07	24
6.t		1.1	25
6.u		K_i = 4.03 μM	11
6.v		R = C_6H_5 20 R = pClC_6H_4 10 R = 4-Pyridyl 2	26
6.w		R = C_6H_5 1.0 R = 4-Pyridyl 0.06 R = pClC_6H_4 0.6	26

Table 6.2 lists some compounds that, according to the models of Robins et al. [8], would have a type II binding with xanthine oxidase (Figure 6.3).

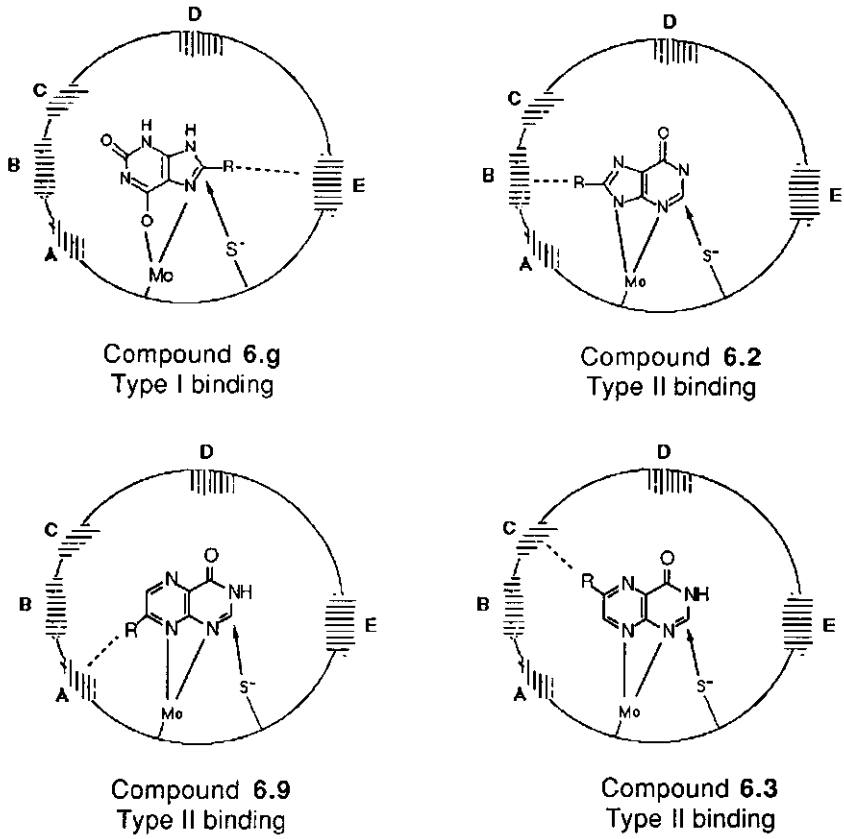
All of these compounds are actually substrates, although some of them, namely **6.2** [5], **6.3** [3], and **6.8** [9], are oxidized only slowly by xanthine oxidase. For compounds **6.2** and **6.3**, an interaction with the wall of the active site is assumed because the different aryl substituents have a large influence on inhibitory capacity. The hydrophobic character of this wall is underscored by the absence of electronic parameters in the QSAR equations for compounds **6.2** and **6.3**. The inhibition of xanthine oxidase seems to be governed mainly by steric factors. Xanthine oxidase favors bulky substituents at the **para** position of the aryl group, and there is an additional requisite for compound **6.3**, namely the substituent X must be rod-shaped. There are two

Table 6.2 Azaheterocyclic Compounds That Have a Type II Binding with Xanthine Oxidase

No.	Compound	Inhibitor	Substrate	Ref.
6.2		yes	C-2	16
6. 3		yes	C-2	22
6.6		no	C-2; C-8	27
6.7		?	C-2; C-7	11
6.8		yes	C-2	11
6.9		no	C-2	28
6.10		?	C-2; C-7 or C-8	10

slight indications of this for compound **6.2**: the hypermolecule in Figure 5.4 shows that positions 2 and 7 are favorable and that they lead to a rod-shaped substituent. Thus it is evident that one cannot make a definitive statement on the nature of the hydrophobic wall based on the congeneric series presented in this thesis.

Figure 6.4 shows how different aryl substituents present at different positions in an inhibitor can interact with the hydrophobic wall of the



Compound **6.g**
Type I binding

Compound **6.2**
Type II binding

Compound **6.9**
Type II binding

Compound **6.3**
Type II binding

Figure 6.4 The molybdopterin active site of xanthine oxidase. A, B, C, D, and E are points in the hydrophobic pocket of the active site with which an aryl substituent (R) of the inhibitor interacts.

active site and how the interaction of the aryl group depends on the type of binding.

For example, the 8-arylxanthines (**6.g**) have a type I binding, which means that the aryl group interacts with part E of the hydrophobic wall of the active site, and the 8-arylhypoxanthines (**6.2**) have a type II binding, which means that the aryl group will interact with part B. This explanation would clarify the difference between the I_{50} values of the 8-arylhypoxanthines and those of the 8-arylxanthines. Other examples are the 6-arylpteridin-4-ones (**6.3**) and the 7-arylpteridin-4-ones (**6.9**), both of which have a type II binding. The aryl group of compound **6.3** interacts with part C and that of compound **6.9** interacts with part A. In addition, one can say that the hydrophobic interaction of the 9-arylguanines (**6.c**) is entirely different from that of the 8-arylhypoxanthines. The 9-arylguanines have a type I binding, which means that the aryl substituent interacts with the hydrophobic wall at point D.

Figure 6.4 shows clearly that all these compounds, which appear to be structurally similar, interact very differently with the active site. The figure also shows that, with the compounds presented in this thesis, one is probing different parts of the active site. While the binding models of Robins et al. will probably not be the final proposal for the molybdenum active site of xanthine oxidase, they at least offer a reasonable explanation of the difference in the I_{50} values of seemingly similar compounds. Further research is needed to understand better the reactions catalyzed by xanthine oxidase.

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SUMMARY

This thesis contains the results of a QSAR analysis of the interaction of bovine milk xanthine oxidase with two azaheterocyclic compounds, namely the 6-arylpteridin-4-ones and the 8-arylhypoxanthines. Xanthine oxidase has active sites for various substrates. The studies done for this thesis were of the active site connected to the molybdenum cofactor.

Chapter 2 contains a description of how the 6-arylpteridin-4-ones and the 8-arylhypoxanthines were prepared. To synthesize the congeneric series of pteridines, the Gabriel-Isay method was used. This method, in which 4,5-diamino-6-hydroxypyrimidin reacts with an arylglyoxal, invariably led to contamination of the reaction product with isomeric 7-arylpteridin-4-ones. Multiple recrystallization from a DMSO-water solution minimizes contamination of the product to less than 5%. To synthesize the 8-arylhypoxanthines, benzoic acid derivatives were used instead of arylglyoxal derivatives.

The 6-arylpteridin-4-ones are good inhibitors of both free and immobilized xanthine oxidase (Chapter 3). To study the inhibitory properties of these compounds as expressed by their K_i values, a QSAR equation was calculated. The equation shows that the electronic character of the 6-arylpteridin-4-one substituents does not influence the inhibition of xanthine oxidase; it is governed only by steric factors. The QSAR equation also shows that the effectiveness of an inhibitor is reduced by large spherical substituents like *t*-butyl and enhanced by rod-shaped substituents like *n*-butyl. For immobilized xanthine oxidase, an additional factor, namely the hydrophobic parameter π, is essential for the equation. The explanation for this lies in the hydrophilic character of the Sepharose matrix that is used to immobilize the enzyme.

The 8-arylhypoxanthines were synthesized and tested for their effectiveness as substrates of xanthine oxidase (Chapter 4). During synthesis, the 8-arylhypoxanthines are converted into the corresponding 8-arylxanthines. To characterize the product, the 8-phenylhypoxanthine was incubated with immobilized xanthine oxidase. The product of the enzymatic reaction was then isolated and characterized as 8-phenylxanthine by ^{13}C-NMR and IR spectroscopy. The oxidation of these compounds is so slow that a detailed study was done only for the unsubstituted compound, the **p**-methyl, and the **p**-bromo substituted 8-arylhypoxanthines. At a high pH

level, V_{max} is considerably higher than at a low level and K_m remains essentially the same. V_{max} and K_m are both higher at high temperatures.

Chapter 5 contains the results of experiments on the inhibition of free xanthine oxidase by the 8-arylhypoxanthines. At very low concentrations, the 8-arylhypoxanthines are effective inhibitors of xanthine oxidase. Consequently, the enzymatic oxidation of the 8-arylhypoxanthines (described in Chapter 4) heavily influences their resulting effective concentration. This makes it so difficult to measure the K_i parameters accurately that only the I_{50} values are given here. The 8-arylhypoxanthines inhibit xanthine oxidase at equimolar concentrations, and the 8-arylxanthines have much higher I_{50} values. Therefore, because the I_{50} values of the 8-arylxanthines are similar to those of the 6-arylpteridin-4-ones, the 8-arylhypoxanthines are just as effective at inhibiting xanthine oxidase as the 6-arylpteridin-4-ones.

Chapter 6 contains a comparison of the inhibitory properties of the 6-arylpteridin-4-ones and the 8-arylhypoxanthines with those of several other azaheterocyclic compounds. The flexibility of the active site of xanthine oxidase is confirmed. The site, which contains the molybdenum co-factor, can accommodate compounds of different sizes. Proof of this is found in the inhibition of xanthine oxidase by a small compound like allopurinol, by larger compounds like 6-arylpteridin-4-one or 8-arylhypoxanthine, and by *lin*-naphthoxanthine, which is twice as large as allopurinol.

With these congeneric compound series, one can also study the "wall" of an enzyme's active site. The interaction of the substituents of the 6-arylpteridin-4-ones and the 8-aryl(hypo-)xanthines with the wall of xanthine oxidase's active site seems to be governed only by steric factors. This is because electronic parameters like σ do not appear in the QSAR equations. The type I and type II binding models explain the large differences between the I_{50} values of the 8-arylhypoxanthines and those of the 8-arylxanthines. Although these compounds are structurally similar, their aryl substituent interacts with different parts of the active site.

SAMENVATTING

Dit proefschrift handelt over een QSAR-analyse van de interactie tussen xanthine-oxidase uit koemelk en twee reeksen van azaheterocyclische verbindingen, namelijk de 6-arylpteridin-4-onen en de 8-arylhypoxanthines. Xanthine-oxidase heeft verscheidene reactiecentra beschikbaar voor verschillende soorten verbindingen. Het onderzoek betrof het reactiecentrum met de molybdeen-cofactor.

In hoofdstuk 2 wordt de synthese van de azaheterocyclische verbindingen beschreven. Voor de reeks van 6-arylpteridin-4-onen werd de Gabriel-Isay-methode gebruikt. Bij deze methode, waarin 4,5-diaminopyrimidin-6-on reageert met een arylglyoxaal-derivaat, ontstaat tevens het isomeer 7-arylpteridin-4-on. Door meermaals om te kristalliseren uit een oplossing in DMSO-water was het mogelijk deze verontreiniging terug te brengen tot minder dan 5 procent. Bij de synthese van de 8-arylhypoxanthines werden benzoëzuur-derivaten gebruikt in plaats van arylglyoxaal-derivaten.

De 6-arylpteridin-4-onen zijn goede remstoffen van zowel vrij als geïmmobiliseerd xanthine-oxidase (hoofdstuk 3). De QSAR-vergelijking laat zien dat het elektronische karakter van de substituenten van de 6-arylpteridin-4-onen geen invloed heeft op de remming van xanthine-oxidase. De verschillen in de remmende werking worden slechts bepaald door sterische factoren. De QSAR-vergelijking toont aan dat de remmende werking verlaagd wordt door grote bolvormige substituenten, zoals *t*-butyl, en vergroot door staafvormige substituenten, zoals *n*-butyl. Voor geïmmobiliseerd xanthine-oxidase blijkt een extra factor, de hydrofobe parameter π, essentieel te zijn voor de QSAR-vergelijking. Deze extra factor wordt verklaard door het hydrofiele karakter van de Sepharose-matrix die gebruikt is voor de immobilisatie van het enzym.

De 8-arylhypoxanthines zijn eerst bestudeerd als substraat voor xanthine-oxidase (hoofdstuk 4). In de enzymatische reactie worden 8-arylhypoxanthines geoxideerd tot de overeenkomstige 8-arylxanthines. Om het produkt te karakteriseren is 8-fenylhypoxanthine geïncubeerd met geïmmobiliseerd xanthine-oxidase. Het produkt is geïsoleerd en door middel van ^{13}C-NMR- en IR-spectroscopie gekarakteriseerd als 8-fenylxanthine. De oxidatie van deze verbindingen verloopt bijzonder langzaam. Daarom zijn slechts de ongesubstitueerde en de **p**-methyl- en de **p**-broom-

8-arylhypoxanthines uitvoerig bestudeerd. Verhoging van de pH leidt tot een aanzienlijke toename van V_{max}. Verhoging van de temperatuur leidt tot een toename van zowel V_{max} als K_m.

Hoofdstuk 5 bevat de resultaten van het onderzoek naar de remming van vrij xanthine-oxidase met 8-arylhypoxanthines. De 8-arylhypoxanthines remmen xanthine-oxidase aanzienlijk bij zeer lage concentraties. Daardoor heeft de enzymatische oxidatie van de remstof, zoals beschreven in hoofdstuk 4, een grote invloed op de werkelijke concentratie. Dit effect verstoort de nauwkeurige meting van de K_i waarde. Daarom worden in hoofdstuk 5 alleen de I_{50} waarden vermeld. Hoewel 8-arylhypoxanthines het enzym reeds remmen bij equimolaire concentraties, zijn ze uiteindelijk als remstof net zo effectief als 6-arylpteridin-4-onen. De I_{50}-waarden van de 8-arylxanthines zijn vrijwel gelijk.

In hoofdstuk 6 worden de remmende eigenschappen van 6-arylpteridin-4-onen en 8-arylhypoxanthines vergeleken met die van enkele andere azaheterocyclische verbindingen. De plooibaarheid van het actieve centrum van xanthine-oxidase wordt bevestigd, doordat verbindingen, die aanzienlijk in grootte variëren, goed blijken te worden gebonden. Dit wordt aangetoond door de remmende werking van een kleine verbinding als allopurinol, en van grotere verbindingen als de 6-arylpteridin-4-onen en de 8-arylhypoxanthines, en van *lin*-naphthoxanthine dat twee keer zo groot is als allopurinol.

Met deze gelijksoortige reeksen van verbindingen kan men tevens de "wanden" van het actieve centrum van het enzym bestuderen. De interactie van de substituenten van de 6-arylpteridin-4-onen en de 8-aryl(hypo-)xanthines met de wand van het actieve centrum van xanthine-oxidase lijkt alleen bepaald te worden door sterische factoren. Dit komt tot uiting in de afwezigheid van elektronische parameters (σ) in de QSAR-vergelijkingen. De type-I- en type-II-bindingsmodellen verklaren de grote verschillen in I_{50}-waarden voor de 8-arylhypoxanthines en de 8-arylxanthines. Hoewel deze verbindingen een vergelijkbare chemische structuur hebben, is hun arylgroep met verschillende delen van het actieve centrum in contact.

CURRICULUM VITAE

Han S.D. Naeff werd geboren op 10 juni 1954 in Medan (Indonesië). Hij volgde de lagere en de middelbare schoolopleiding in Winterswijk; het diploma HBS-B werd in 1971 gehaald aan de Rijksscholengemeenschap "De Hameland" te Winterswijk. In 1972 werd begonnen met de studie Moleculaire Wetenschappen aan de Landbouwhogeschool te Wageningen. De praktijktijd werd doorgebracht op het Planten Virologisch Laboratorium van het USDA Agrarisch Onderzoekscentrum te Beltsville, Maryland, in de Verenigde Staten, onder de begeleiding van dr. T.O. Diener. In 1980 studeerde hij af als landbouwkundig ingenieur met als hoofdvakken Moleculaire Biologie en Organische Chemie en als bijvak Moleculaire Fysica.

In de periode van september 1980 tot september 1987 werd het in dit proefschrift beschreven onderzoek uitgevoerd onder leiding van prof. dr. H.C. van der Plas. Hij was eerst tot september 1984 als wetenschappelijk medewerker in tijdelijke dienst bij de Stichting Zuiver Wetenschappelijk Onderzoek (ZWO), sectie Scheikundig Onderzoek Nederland (SON) en vervolgens als wetenschappelijk projectmedewerker verbonden aan de vakgroep Organische Chemie van de Landbouwhogeschool te Wageningen. Gedurende het promotieonderzoek was hij betrokken bij het onderwijs aan studenten in verschillende fasen van hun studie en aan HBO stagiaires.