OXIDATION OF N-ALKYL AND N-ARYL AZAHETEROCYCLES BY FREE AND IMMOBILIZED RABBIT LIVER ALDEHYDE OXIDASE

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Proefschrift
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STELLINGEN

1. De conclusie van Jadhav *et al.* dat 6,6'-azopurine een sterke remmer is van aldehyde oxydase wordt onvoldoende onderbouwd met experimentele gegevens.

   A.L. Jadhav, K.G. Bhansali en J.R. Davis, J.Pharm.Sci., 68, 1202 (1979).

2. Het is niet waarschijnlijk dat in het door Bray *et al.* voorgestelde mechanisme voor de oxydatie van xanthine door xanthine oxydase de initiële nucleofiele aanval verloopt op de beschreven wijze.

   R.C. Bray, S. Gutteridge, D.A. Stotter en S.J. Tanner, Biochem.J., 177, 357 (1979).

3. Ter bevordering van de toegankelijkheid van veel moderne (Nederlandse) literatuur is het aan te bevelen in boeken een begeleidend woord op te nemen over de voornaamste complexen en/of trauma's van de auteurs, aangezien de gemiddelde lezer geen opleiding in de psycho-analyse heeft genoten.

4. Bij de beschrijving van de bitterheid van enzymatische eiwithydrolysaten in relatie tot de molekuulgrootte van de peptiden houdt Ney te weinig rekening met de specificiteit van het protease en de verdeling van hydrofobe aminozuren over het substraat.

   K.H. Ney, Fette, Seifen, Anstrichmittel, 80, 323 (1978).
   J.P. Roozen en J. de Groot, Sixth Intern. Congress Food Sci. Technol., Dublin, September 18-23 (1983).

5. De wijze waarop Brandänge en Lindblom K_i-waarden berekenen voor het nicotine-$\Delta^{1'(5')}$-iminiumzout en zijn pseudobase in evenwichtsmengsels van deze componenten, is onjuist.

   S. Brandänge en L. Lindblom, Biochem.Biophys.Res.Commun., 91, 991 (1979).

6. De experimenten van Johnson en Coughlan en Tramper *et al.* sluiten niet uit dat gedeeltelijke inaktivering van xanthine oxydase optreedt gedurende katalyse tengevolge van het verlies van labiel zwavel.

D.B. Johnson en M.P. Coughlan, Biotechnol.Bioeng., 20, 1085 (1978).
J. Tramper, F. Müller en H.C. van der Plas, Ibid., 20, 1507 (1978).
R.C. Wahl en K.V. Rajagopalan, J.Biol.Chem., 257, 1354 (1982).

7. Het verheerlijken van staatshoofden gaat meestal niet samen met een democratische regeringsvorm.

8. De aanwezigheid van hexamethylfosforamide bij de omzetting van 8-chloor-3,10-dimethylpyrimido[4,5-b]chinoline-2,4(3H,10H)-dion met dimethylamine naar 8-dimethylamino-3,10-dimethylpyrimido[4,5-b]chinoline-2,4(3H,10H)-dion is overbodig en zelfs ongewenst.

F. Yoneda, K. Mori, Y. Sakuma en A. Koshiro, J.Heterocyclic Chem., 19, 945 (1982).

9. Er bestaat geen duidelijk verband tussen de suikersamenstelling van pektines en hun geleersterkte.

J.A. de Vries, A.G.J. Voragen, F.M. Rombouts en W. Pilnik, Carbohydr. Polymers, 2, 25 (1982).

10. De beste stelling voor een promovendus is een aanstelling.

Steven Angelino Wageningen, 3 februari 1984

Oxidation of N-alkyl and N-aryl azaheterocycles by free and immobilized rabbit liver aldehyde oxidase.

Kennis zij macht;
geen macht is goed geplaatst,
tenzij er wijsheid zij,
er boven of er naast.

N. Beets

VOORWOORD

Bij de voltooiing van dit proefschrift gaat mijn dank uit naar allen die op enigerlei wijze aan de totstandkoming ervan hebben bijgedragen. Een aantal van hen wil ik hier in het bijzonder noemen.
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CONTENTS

1 INTRODUCTION

1.1 GENERAL

The use of enzymes in organic synthesis has been a research subject in the Departments of Organic Chemistry and Biochemistry since 1975. A group of hydroxylases, belonging to the class of oxido-reductases were chosen as model enzymes because of their broad substrate specificities towards azaheterocycles. The chemistry of the latter is a major topic in the laboratory of Organic Chemistry[1-5]. Representatives of this group of enzymes, xanthine oxidase and xanthine dehydrogenase have been employed succesfully, both in free and immobilized form, in laboratory scale oxidations of azaheterocyclic compounds[6-11]. Furthermore, the application of free and immobilized bovine milk xanthine oxidase in the development of anti-gout drugs was and remains an important area of research[12,13]. To date aldehyde oxidase, another hydroxylase within this group of enzymes, has not been studied for its applicability in synthetic organic chemistry. The enzyme possesses a markedly different substrate specificity compared to xanthine oxidase and xanthine dehydrogenase. The utilization of this enzyme should therefore greatly increase the synthetic prospects of this group of biocatalysts. This thesis presents a study on the use of rabbit liver aldehyde oxidase in organic synthesis.

1.2 IMMOBILIZED ENZYMES IN SYNTHETIC ORGANIC CHEMISTRY

The application of enzymes in synthetic organic chemistry offers enormous potential, a fact already recognized for a long time. The first examples of profitable use of biocatalysts have been reported in the early fifties[14]. In general the most important feature of enzymes is their potential ability to catalyze reactions which are chemically difficult or impossible to perform.
Other important aspects of a more practical nature of the use of enzymes, such as a fast reaction rate, a high reaction specificity and mild reaction conditions, are also very propitious.
In spite of the promising prospects, the application of enzymes in this area has progressed rather slowly in the past thirty years. This was due to numerous factors such as availability of purified enzymes in sufficient amounts, instability of isolated enzymes and limited recovery of functional enzyme from usually

aqueous reaction mixtures. Accordingly, an efficient utilization of enzymes was very desirable, but hardly possible.

The immobilization of enzymes proved to be a proper solution to overcome many of these problems[15]. As most important advantages of immobilized against soluble enzymes we stress the possibility of repeated use, their greater stability, the feasibility of a continuous mode of operation, the formation of less contaminated products and a minimal work-up.

In the past ten years the interest in and the efforts towards the application of immobilized enzymes and microbial cells in organic synthesis has increased substantially, and general criteria for the acceptance of an immobilized biocatalyst have been formulated[16]. The documentation and availability of purified enzymes have been improved and many immobilization techniques using various supports were developed[15,17-20]. Special fields of interest for the application of immobilized enzymes/microbial cells are found at present in the synthesis of antibiotics, steroids, optically pure amino acids and peptides[21-24].

Although much more is known nowadays about the theoretical aspects of enzyme immobilization and stabilization, the best choice of a support/immobilization method combination for a specific enzyme (or microbial cell) still has to be made by a process of trial and error. Furthermore, the demands set by the system under study will always dictate the choice of the approach[15].

1.3 ALDEHYDE OXIDASE

Aldehyde oxidase (E.C.1.2.3.1) along with xanthine oxidase (E.C.1.2.3.2) and xanthine dehydrogenase (E.C.1.2.1.37) belongs to a group of closely related molybdenum iron-sulfur containing flavoproteins. The reaction catalyzed by these enzymes can formally be described as a hydroxylation.

$$RH + H_2O \longrightarrow ROH + 2e^{\ominus} + 2H^{\oplus}$$

In this schematic representation RH is the substrate to be oxidized and the oxygen introduced into RH is not derived from molecular oxygen but from water. This aspect distinguishes these enzymes from other types of hydroxylases. The physiological electron acceptor is molecular oxygen in the case of the oxidases and coenzyme $NAD^{\oplus}$ for the dehydrogenase, which are reduced to H_2O_2 and NADH, respectively. However, many other compounds can serve as an artificial electron acceptor for these enzymes, both under aerobic and anaerobic conditions[25-29]. Biochemical studies of this class of enzymes have mainly been concerned with xanthine oxidase and xanthine dehydrogenase. The former was usually isolated from bovine milk and the latter from avian livers[25].

Aldehyde oxidases have been investigated far less extensively. The enzyme isolated from rabbit liver is as yet the best studied among the mammelian aldehyde oxidases. This is probably due to both the easy availability of rabbit livers and the relatively high level of aldehyde oxidase activity in the livers[30]. Rabbit liver aldehyde oxidase is located in the cytoplasm of the cells[31] and can be isolated in a rather simple manner[31-34]. Table 1.1 summarizes some properties of highly purified rabbit liver aldehyde oxidase and bovine milk xanthine oxidase and shows the similarities between both enzymes.

Table 1.1 Composition and spectral properties of rabbit liver aldehyde oxidase (AO) and bovine milk xanthine oxidase (XO)

Enzyme	Mol.wt.	A_{280}/A_{450}	$A^{1\%}_{1\ cm}(280)$	ε_{450} (per 2 FAD)	Mol/mol FAD Mo	Fe	S	Ref.
AO	280,000	-	-	63,000	1	4	-	31,35
AO	270,000	5.2	12.4	-	0.33-0.57[a]	3.96	-	33
AO	346,000	5.5	-	69.400	0.87	4.10	-	34
XO	283,000	5.0	11.7	72.000	1	4	4	36
XO	362,000	5.4	-	76,000	1	4	4	37
XO	303,000	4.8	-	72.500	-	-	-	38

a. Low values because of molybdenum loss during purification[33].

Like the other hydroxylases in this group aldehyde oxidase consists of two equivalent but independent subunits and contains one atom of molybdenum, one molecule of flavin and two spectroscopically distinct Fe_2S_2 centres per subunit[39,40]. The molybdenum centre of aldehyde oxidase contains a pterin cofactor, as found in xanthine oxidase/dehydrogenase[40]. The function of this cofactor is still not understood.

In its functional form the molybdenum centre of aldehyde oxidase possesses a cyanolyzable sulphur atom[34]. Removal of this sulphur atom gives inactivation of the enzyme, which can be reversed by incubation of the desulfo enzyme with sodium disulfide and dithionite under anaerobic conditions[41]. Branzoli and Massey[34,42] provided evidence that the oxidation of substrates occurs at the molybdenum centre, while reduction of oxygen takes place at the FAD, a reaction sequence analogous to the mechanism of xanthine oxidase[43,44].

Further proof for the close structural relationship between rabbit liver aldehyde oxidase and bovine milk xanthine oxidase was presented recently by EPR and potentiometric studies[40,45-47]. This work suggests that the striking differences in substrate specificity for both enzymes must be primarily related to structural differences of the active centre with regard to the substrate binding site, not involving the catalytically important molybdenum site[46].

A kinetic and chemical model for the catalysis by aldehyde oxidase has not yet appeared in the literature but for the time being the model presented by Olson *et al.*[44] for bovine milk xanthine oxidase seems to be a fair approximation because of the close structural relationship (Figure 1.1). The basic concept of this model will, therefore, be used in the interpretation of our results obtained with rabbit liver aldehyde oxidase.

The model proposes a chemical mechanism in which the substrate is attacked by a nucleophile, in actual fact a persulfide group, in the Michaelis complex I.

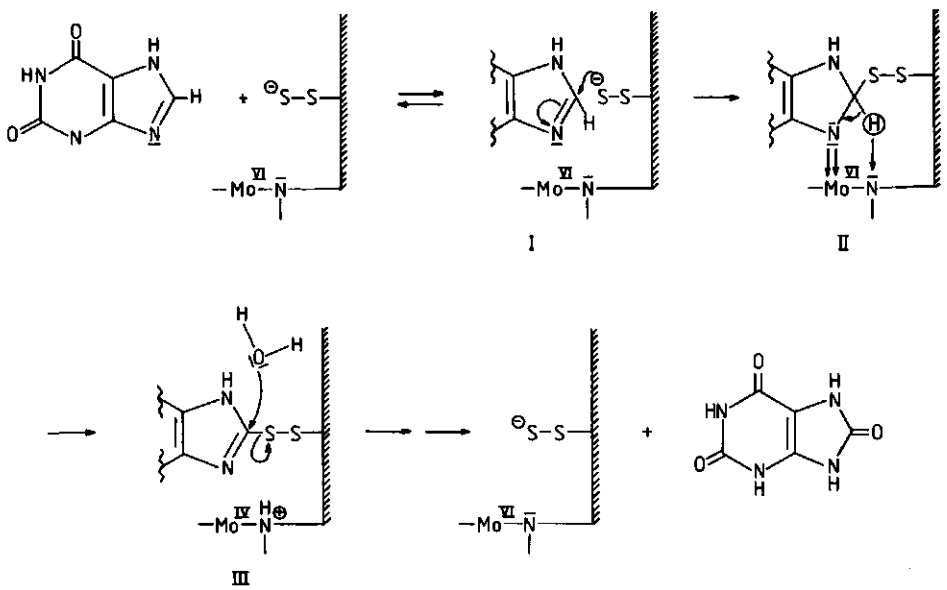

Figure 1.1 Representation of the oxidation of xanthine by xanthine oxidase as proposed by Olson et al.[44]. Complex II is introduced as additional intermediate complex to make the model chemically more feasible.

Subsequent rehybridization of the sp^3-carbon to a sp^2-carbon by proton abstraction and electron transfer then takes place (II), and finally the persulfide linkage to the reaction intermediate is hydrolyzed by a water molecule with release of the product (III). It is still not clear whether the electron transfer actually occurs as a coupled proton/electron transfer or as a hydride transfer[25]. Various other proposals have been made for the nature of the nucleophilic species[48-51] and of the proton acceptor[25,48,51], although none of these modifications basically alter the concept of the mechanism presented. Some evidence that pteridine substrates can react with the active site molybdenum producing a catalytically important charge-transfer complex has recently been presented for xanthine oxidase[52].

1.4 SUBSTRATE SPECIFICITY OF ALDEHYDE OXIDASE AND XANTHINE OXIDASE

Aldehyde oxidase catalyzes, like xanthine oxidase and xanthine dehydrogenase, the hydroxylation of a wide variety of purines, pteridines, pyrimidines, other heterocyclic nitrogenous compounds and aliphatic, aromatic or heteroaromatic aldehydes[25,53-56]. The rates at which these compounds are oxidized by these enzymes differ widely.

Table 1.2 Effects of a second C-substituent on the rates of oxidation of azaheterocycles by aldehyde oxidase (AO) and xanthine oxidase (XO)

Substrate	Initial rate of oxidation[a]	
	AO	XO
6-mercaptopurine	16	17
2-hydroxy-6-mercaptopurine	<1	42
2-hydroxypurine	140	53
2,4-dihydroxypurine	<1	170
6-amino-2-hydroxypurine	<1	27
4-hydroxypteridine	164	86
2,4-dihydroxypteridine	<1	80

a. Arbitrary units.

Comparison of rabbit liver aldehyde oxidase and bovine milk xanthine oxidase shows the following most distinct differences:

a. Both enzymes readily oxidize a variety of unsubstituted and C-monosubstituted azaheterocycles, but the introduction of a second C-substituent always decreases the activity of aldehyde oxidase, while the activity of xanthine oxidase usually remains unaffected and, in some cases actually increases (Table 1.2)[53].

b. Introduction of an alkyl substituent at a ring nitrogen often obliterates the activity of xanthine oxidase, whereas in many cases aldehyde oxidase exhibits an increased activity. The effects of N-methylation on the substrate activity of hypoxanthine with both enzymes are compared in Table 1.3 as an example[53].

Table 1.3 Effects of N-methylation on the substrate activity of hypoxanthine with aldehyde oxidase (AO) and xanthine oxidase (XO)

Position of N-methylation of hypoxanthine	Initial rate of oxidation[a] AO	XO
none	3	130
N-1	34	19
N-3	710	<3
N-7	27	4
N-9	2	<3

a. Arbitrary units.

N-Alkylazinium salts are usually more reactive than the corresponding azines, which is reflected by the increased rate of oxidation of e.g. 3-pyridinecarboxamide or quinoline by aldehyde oxidase upon N-methylation[53].

c. The position of hydroxylation in the substrate can differ substantially for both enzymes. Furthermore with substrates such as purines and pteridines, which have more than one site available for oxidation, xanthine oxidase often performs a sequence of hydroxylation reactions, whereas aldehyde oxidase frequently exhibits a high site selectivity. In the oxidation of purine for example, the substrate is converted to uric acid in three successive steps by xanthine oxidase, whereas aldehyde oxidase oxidizes purine to 8-hydroxypurine[25]. It has been reported however, that isomeric products are formed with some substrates in the oxidation of N-alkylazinium compounds by aldehyde oxidase[33,57].

1.5 OUTLINE OF THIS THESIS

In general the hydroxylation of N-alkyl(aryl)azaheterocycles by rabbit liver aldehyde oxidase is scarcely documented and very little information is available on the factors determining the site of oxidation. The aim of this study is elucidation of the effect of N-alkyl and N-aryl substituents in relation to steric and electronic factors on the site of oxidation in these compounds. Special attention is paid to the formation of isomeric products in the oxidation of N-alkyl(aryl)azinium salts.

In context with this study it is investigated whether immobilized aldehyde oxidase can be profitably applied in synthetic organic chemistry.

The oxidation of 1-alkyl-3-aminocarbonylpyridinium chlorides is reinvestigated in chapter 2, especially with regard to the occurrence of isomeric oxidation products[58].

This aspect is studied in more detail on description of the aldehyde oxidase-mediated oxidation of the 1-aryl analogues in chapter 3. Important information is presented on the rate-limiting step in the oxidation mechanism of aldehyde oxidase. A comparison between the action of bovine milk xanthine oxidase and aldehyde oxidase on these substrates is made[59].

Chapter 4 deals with covalent addition of 1-alkyl(aryl)-3-aminocarbonylpyridinium chlorides as a potential model for the covalent addition step in the enzymic oxidation mechanism[60].

The oxidation of 1-alkyl(aryl)quinolinium chlorides by aldehyde oxidase and comparison to the covalent amination model is presented in chapter 5[61].

Chapter 6 describes the oxidation of 1-methyl- and 1-benzylpyrimidin-2- and -4-ones by aldehyde oxidase[62].

Chapter 7 comprises the immobilization of aldehyde oxidase by various methods and their utility in terms of (operational) stability and productivity[63].

A general discussion on the catalytic centre of aldehyde oxidase based on the work in this thesis supplemented with miscellaneous results is given in chapter 8.

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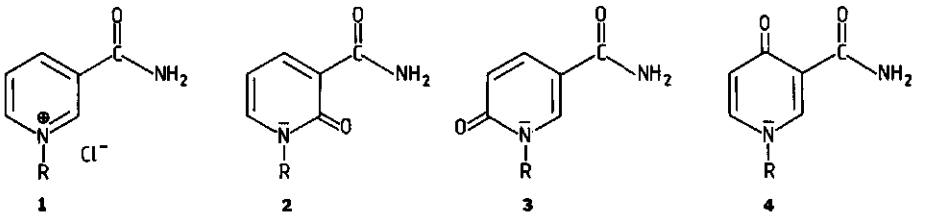
2 THE OXIDATION OF 1-ALKYL-3-AMINOCARBONYL-PYRIDINIUM CHLORIDES BY ALDEHYDE OXIDASE

2.1 INTRODUCTION

In our laboratories we are interested in the use of immobilized enzymes for the synthesis of heterocyclic compounds on a preparative scale[1-5]. In this study we report on the oxidation of 1-alkyl-3- aminocarbonylpyridinium chlorides 1 by free and immobilized rabbit liver aldehyde oxidase (Scheme 2.1). The oxidation of these pyridinium salts 1 by alkaline ferricyanide has been described[6,7]. From the 3- aminocarbonyl-1-methylpyridinium salt 1a two products, *i.e.* 1,2-dihydro-1-methyl-2-oxo-3-pyridinecarboxamide (2a) and its 6-oxo isomer (3a), were derived in a ratio of about 1 : 1; no production of the 4-oxo isomer (4a)



R = a: CH_3 , b: C_2H_5 , c: n-C_3H_7, d: i-C_3H_7 , e: t-C_4H_9

Scheme 2.1

was observed. In contrast, the oxidation of 1a by rabbit liver aldehyde oxidase was reported to yield a mixture of 6-oxo (3a) and 4-oxo isomers (4a) in a ratio of about 100 : 1; no indication for the presence of the 2-oxo isomer (2a) was found[8,9]. When the *n*-propyl derivative 1c was used as substrate, the ratio of products 3c and 4c decreased to 4.1 : 1, indicating that an increase in the size of the alkyl substituent at position 1 in compounds 1 makes oxidation at position 4 more favourable[9]. Initial experiments in our laboratories concerning the oxidation of 1a by aldehyde oxidase showed no formation of the 4-oxo compound (4a) at all and this result, being in contradiction to the reported results, induced us to investigate the enzymic oxidation reaction of 1-alkyl-3-aminocarbonylpyridinium salts 1a-e with free and immobilized aldehyde oxidase in more detail. Special attention was paid to the effect of alkyl substituents on

product ratios with respect to the hydrophobicity of the substituents and the possible steric hindrance caused by increasing the size of these substituents. A possible application of immobilized aldehyde oxidase as oxidizing agent for laboratory-scale preparations was also considered.

2.2 RESULTS AND DISCUSSION

As already mentioned in the introduction incubation of compound 1a with aldehyde oxidase gave the 6-oxo compound (3a) as single product. This result was found by analysis of the reaction mixture with HPLC, utilizing conditions in which separation of 2a, 3a and 4a could be achieved, as shown by the use of independently prepared samples[6,10]. Similarly in the enzymic oxidation of the 1-ethyl and 1-*n*-propyl salts 1b, 1c, only formation of the corresponding 6-oxo compounds 3b, 3c was observed.

The fact that the enzymic reaction resulted in only one product and not in a mixture of products prompted us to investigate whether 1-alkyl-1,6-dihydro-6-oxo-3-pyridinecarboxamides 3 could be prepared on a small laboratory scale (about 100 mg) by oxidation with immobilized aldehyde oxidase. As substrate we used 3-aminocarbonyl-1-*n*-propylpyridinium chloride (1c). The reaction appeared to occur rather slowly: a few weeks at 4°C resulted in conversion of 1c into 3c in about 85% yield. The structure of the product was established by comparison of the ^{1}H NMR data (Table 2.1) and mass spectrometric data with those of an authentic specimen, obtained by oxidation of 1c with alkaline ferricyanide. Although 3c can be acquired in a pure state by using immobilized aldehyde oxidase, the long reaction time is a disadvantage of this method of preparation.

The enzymic oxidation of 3-aminocarbonyl-1-isopropylpyridinium chloride (1d) yielded two products as shown by HPLC, but from 3-aminocarbonyl-1-*tert*-butylpyridinium chloride (1e) only one product was obtained. Since no reference compounds of any of the 1-isopropyl- or 1-*tert*-butyldihydro-oxo-3-pyridinecarboxamides are known, we prepared the products obtained in the enzymic reactions on a preparative scale using immobilized aldehyde oxidase in order to determine their structures.

GC-MS analysis of the reaction mixture acquired from compound 1d revealed that both products are isomeric 1-isopropyldihydro-oxo-3-pyridinecarboxamides. After isolation we were able to unambiguously establish the identity of the two products as the 6-oxo (3d) and the 4-oxo isomer (4d). These structural assignments were based on UV and ^{1}H NMR spectroscopic data. The UV spectrometric method is based on the experimental finding that the spectrum of 3d is almost pH

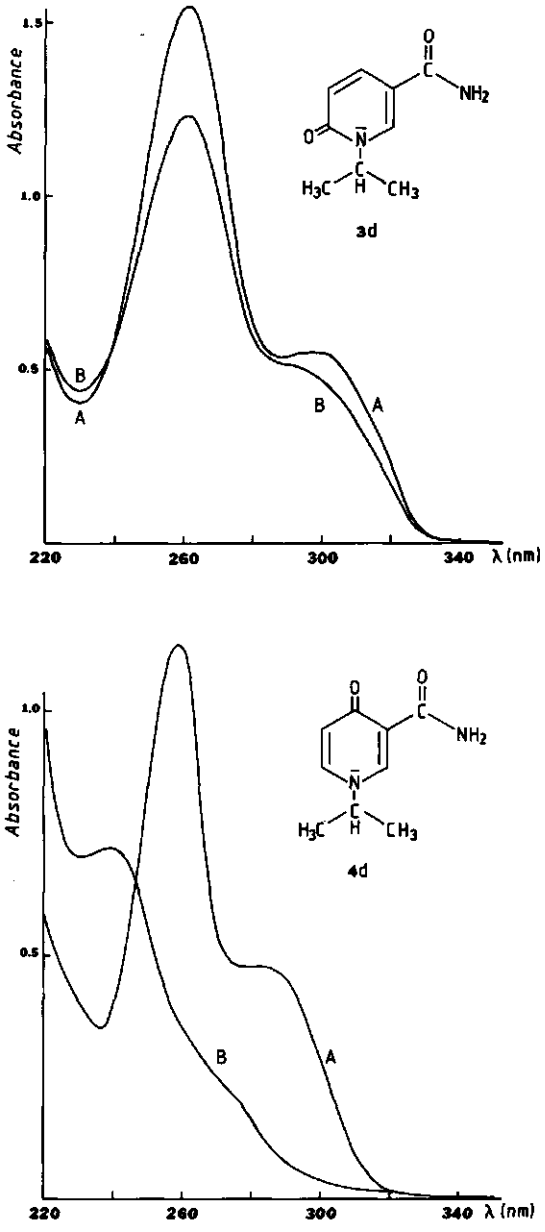


Figure 2.1 UV spectra of the enzymic oxidation products of 1d in water (A) and 1N HCl (B); 3d: 0.14 mM (A), 0.12 mM (B); 4d: 0.10 mM (A), 0.09 mM (B).

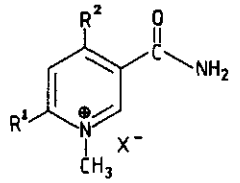
independent while that of 4d is pH dependent (see Figure 2.1).This behaviour has been found to be of diagnostic value for differentiating 4- and 6-oxo compounds[11]. Confirmation of the structures 3d and 4d was obtained by ^{1}H NMR spectroscopy using the difference in magnitude of the *ortho* and *meta* coupling constants as found in the reference compounds 3a ($J_{4,5}$= 9.2 Hz and $J_{2,4}$= 2.7 Hz) and in 4a ($J_{5,6}$= 7.5 Hz and $J_{2,6}$= 2.4 Hz; see Table 2.1).

Table 2.1 Chemical shifts and coupling constants of the ring protons of compounds 3a-d, 4a and 4d-e in CD_3OD

Compound	H-2	H-4	H-5	H-6	$J_{2,4}$	$J_{4,5}$	$J_{2,6}$	$J_{5,6}$
3a	8.30	7.90	6.50		2.7	9.2		
3b	8.25	7.79	6.53		2.7	9.2		
3c	8.20	7.84	6.55		2.7	9.2		
3d	8.33	7.92	6.53		2.7	9.2		
4a	8.51		6.53	7.72			2.4	7.5
4d	8.68		6.65	7.98			2.4	7.5
4e	8.82		6.60	8.14			2.4	7.5

From the peak heights in the HPLC pattern of the reaction mixture a product ratio 3d/4d of approximately 3.5 : 1 was calculated.
The product isolated from the reaction of 1e with immobilized aldehyde oxidase, using the same methods was shown to be 1-*tert*-butyl-1,4-dihydro-4-oxo-3-pyridinecarboxamide (4e). The results of our experiments seem to indicate that increasing the size of the 1-alkyl group increases the steric hindrance to oxidation at position 6 of the ring, making oxidation at position 4 more favourable or the only alternative. In the case of the *tert*-butyl group, the latter seems to be the only possible reaction.
Experiments using 3- aminocarbonyl-1-methylpyridinium salts (Scheme 2.2) showed that methyl substituents at position 6 and/or 4 prevent the formation of oxo-3-pyridinecarboxamides. When position 6 is substituted by a methyl group, we found that 5a and also the 4,6-dimethyl compound 5c gave no product. From the 4-methyl derivative 5b we obtained, in a very slow reaction, a trace of a single

product (as observed by HPLC) which is probably the corresponding 6-oxo compound.



a: $R^1 = CH_3$, $R^2 = H$, $X = Cl^-$
b: $R^1 = H$, $R^2 = CH_3$, $X = I^-$
c: $R^1 = CH_3$, $R^2 = CH_3$, $X = I^-$

5

Scheme 2.2

It has been suggested[9] that the formation of the 4-oxo derivatives in the enzymic reaction could be due to a different binding orientation of the substrate. We examined this hypothesis more closely by determining the kinetic constants V and K_M for some of these substrates. Assuming simple Michaelis-Menten kinetics[12], we obtained the results summarized in Table 2.2. As one can see, the maximum rate V drops rather drastically to about 6% when the 1-alkyl substituent is changed from methyl to ethyl to n-propyl. The very low rates for 1d and 1e (the oxidation rate for 1d is about 7% of that of 1c at a substrate concentration of 0.8 mM) prevent accurate kinetic assays with these compounds. We assume that the mechanism for oxidation by aldehyde oxidase is analogous to that proposed by Olson *et al.*[13] for the oxidation of xanthine by bovine milk xanthine oxidase, *i.e.* an initial nucleophilic attack at position 6 of the substrate (1a-c). This position is the most suitable for nucleophilic attack, as can also be seen from the experiments with the 4- and/or 6-methyl derivatives 5.
The considerable drop in oxidation rate observed in going from 1a to 1c cannot be explained simply by a decreased reactivity towards nucleophilic attack resulting from the somewhat more electron-donating character of the ethyl and n-propyl substituents since the effective size of these groups must also play a role. The Michaelis constant K_M decreases with increasing size of the alkyl substituent suggesting that the association of the enzyme-substrate complex for compounds 1b and 1c increases with larger hydrophobic substituents. These results point to the existence of a hydrophobic site in the vicinity of the active centre of rabbit liver aldehyde oxidase; this proposal parallels that of Baker *et al.*[15] for bovine milk xanthine oxidase.
To obtain further support for this proposal, we performed inhibition experiments utilizing 1b-e as inhibitors of the oxidation reaction of 1a. The inhibition constants K_i[12] are given in Table 2.2. We established competitive inhibition

which increased with the size of the alkyl substituent at position 1, pointing to a growing affinity of the enzyme for these compounds (1b,1c).

Table 2.2 Kinetic data for the oxidation of compounds 1a-d by free aldehyde oxidase at pH=9.0

Compound	K_M [a]	V [b]	K_i [a,c]
1a	310 ± 14	0.330 ± 0.020	
1b	100 ± 6	0.020 ± 0.002	124 ± 6
1c	14 ± 1	0.020 ± 0.003	21 ± 2
1d	-	-	100 ± 6
1e	-	-	39 ± 3

a. In µmol/l.

b. In µmol/min.mg.

c. The small contributions of the rate of the oxidation of the inhibitors to the oxidation rate of 1a were neglected.

When a branched alkyl group was present (1d,1e), the competitive inhibition decreased [K_i(1d) *versus* K_i (1c)], although 1e gave rise to greater inhibition in this reaction than did 1d. These results seem to support the hypothesis that, due to affinity of the hydrophobic site close to the active centre of the enzyme and due to the size of the *tert*-butyl substituent, the binding orientation has changed in such a way that attack of the nucleophilic group is only possible on the C-4 position.

2.3 EXPERIMENTAL SECTION

Melting points are uncorrected. ^{1}H NMR spectra were measured using an Hitachi Perkin Elmer R-24B or a Varian EM 390 spectrometer, with TMS or DSS as internal standard (δ = 0 ppm). The mass spectra were recorded on an AEI MS 902 instrument and GC-MS analysis was performed on a VG-Micromass 7070F apparatus, equipped with a 3% OV-17 column (programmed 170-240°C, 6°C/min). UV spectra were determined using an Aminco DW-2a UV-VIS spectrophotometer. HPLC analysis was performed using a Varian 5000 instrument equipped with a Micro Pak MCH-10 column (30 x 0.4 cm), a Schoeffel GM 770 monochromator and a SF 770 spectroflow monitor. Operating conditions were: Δp = 17.2 MPa (2587 psi), eluent water/methanol 70/30 (v/v) and flow rate 2.0 ml/min. For preparative HPLC a Polygosil 60D 10C_{18} column (25 x

0.9 cm) was used. Column chromatography was carried out over Merck Silica gel 60 (70-230 mesh ASTM). CNBr-activated Sepharose 4B and DEAE Sepharose CL 6B were purchased from Pharmacia Fine Chemicals. Hydroxylapatite (Bio-Gel HT) was obtained from Bio-Rad.

Preparation of starting materials

1-Methyl-, 1-ethyl- and 1-n-propyl-3-aminocarbonylpyridinium chlorides (1a-c) were prepared from their iodide or bromide salts[16] by passage over a Dowex 1-X2 column. *1,2-Dihydro-1-methyl-2-oxo-3-pyridinecarboxamide (2a)*[6] and *1,4-dihydro-1-methyl-4-oxo-3-pyridinecarboxamide (4a)*[10] were synthesized using known synthetic procedures.

3-Aminocarbonyl-1-isopropylpyridinium chloride (1d)

3- Aminocarbonyl-1-(2,4-dinitrophenyl)pyridinium chloride[17] (6.5 g, 20 mmol) was dissolved in 30 ml of methanol and 3.5 g of isopropylamine was added. After heating for 10 min at 40-45°C, the precipitate (2,4-dinitroaniline) was filtered off and ether was added to the filtrate until no further precipitation occurred. Filtration and recrystallization of this residue from ethanol/ether gave 2.2 g of 1d (yield 55%); m.p. 245-247°C. Anal.calcd. for $C_9H_{13}ClN_2O$: C,53.86, H, 6.41; found: C,53.87, H, 6.53.

3-Aminocarbonyl-1-tert-butylpyridinium chloride (1e)

This compound was prepared by treatment of 3- aminocarbonyl-1-(2,4-dinitrophenyl)pyridinium chloride[17] with *tert*-butylamine using the same procedure as described above. Yield: 57%; m.p. 221-222°C (lit.[18] 201°C). Anal. calcd. for $C_{10}H_{15}ClN_2O$: C,55.94, H,7.04; found: C,56.22, H,6.94.

3-Aminocarbonyl-1,6-dimethylpyridinium chloride (5a)

6-Methyl-3-pyridinecarboxamide[19] (0.5 g, 3.7 mmol) was refluxed for 4 h with an excess of methyl iodide in 20 ml of acetophenone. After evaporation of the solvent and recrystallization of the residue from ethanol/ether, 0.93 g of 5a (as iodide) was obtained (yield 90%). This compound was converted into the chloride salt 5a using the method described above. M.p. 265-266°C. Anal. calcd. for $C_8H_{11}ClN_2O$: C,51.48; H, 5.94; found: C,51.35, H,6.04.

3-Aminocarbonyl-1,4-dimethylpyridinium iodide (5b)

4-Methyl-3-pyridinecarboxamide[20] was methylated to 3-aminocarbonyl-1,4-dimethylpyridinium iodide, using the same procedure as described for 5a. Yield 83%; m.p. 214.5-216.5°C. Anal. calcd. for $C_8H_{11}JN_2O$: C,34.55, H,3.99; found: C,34.37, H,4.00.

3-Aminocarbonyl-1,4,6-trimethylpyridinium iodide (5c)

Ethyl 4,6-dimethylpyridine-3-carboxylate[21] (1.5 g, 8.4 mmol) was added to 25 ml of conc. ammonia. After standing for 24 h at room temperature, a precipitate was formed. This precipitate was treated with a cold solution of sodium carbonate; 4,6-dimethyl-3-pyridinecarboxamide was obtained by filtration. The isolated product was converted into 5c by refluxing with an excess of methyl iodide. After recrystallization from ethanol/ether, 0.94 g of 5c was obtained; yield 37%; m.p. 168-170°C. Anal. calcd. for $C_9H_{13}JN_2O.\frac{1}{2}H_2O$: C,35.90, H,4.69; found: C,35.93, H,4.97.

1-Alkyl-1,6-dihydro-6-oxo-3-pyridinecarboxamides (3a-c)

1-Methyl-(3a),1-ethyl- (3b) and 1-*n*-propyl-1,6-dihydro-6-oxo-3-pyridinecarboxamide (3c) were obtained in a small yield by oxidation of 1a, 1b and 1c, respectively with alkaline ferricyanide using the procedure of Pullman and Colowick[6]. The crude products were purified by column chromatography, eluting with chloroform/methanol (30:1).
3a: m.p. 213-215°C (lit.[6] 213-215°C); 3b: m.p. 159-160°C. Anal. calcd. for $C_8H_{10}N_2O_2$: C,57.82, H,6.07; found: C,57.64, H,6.02; 3c: m.p. 197-198°C. Anal. calcd. for $C_9H_{12}N_2O_2$: C,59.98, H,6.71; found: C,59.83, H,6.71.

Enzyme isolation

Rabbit liver aldehyde oxidase (E.C. 1.2.3.1) was partially purified by performing four of the six purification steps described by Felsted *et al.*[9]. Instead of calcium phosphate gel, hydroxylapatite was used and the enzyme was eluted from this material with a linear gradient of 0-200 mM potassium phosphate buffer, pH=7.8 containing 0.1 mM EDTA. Fractions with main aldehyde oxidase activity were combined and the protein was precipitated by addition of ammonium sulphate (0.60 saturation). After centrifuging, the material was resuspended in Tris-HCl (I=0.01) pH=7.8, containing 0.1 mM EDTA, dialyzed and stored at -25°C until required.

HPLC analysis

Substrates were incubated with aldehyde oxidase (protein concentration about 8 µg/ml) at a concentration of 2mM in Tris-HCl, pH=7.8, containing 0.1 mM EDTA at 4°C for several weeks. The course of the reactions was followed by direct injection of 6 µl of the reaction mixtures into the HPLC ststem. Registration of the oxidation products was carried out by UV detection at 254 nm (log ε for 2a: 3.00[6]; for 3a: 4.04; for 3b: 4.08; for 3c: 4.12; for 4a: 3.97).
Each week, fresh enzyme was added to the incubation mixtures to promote the conversion reactions. Product identification was carried out by comparison with authentic materials.

Preparative scale conversion with aldehyde oxidase

Immobilization. Aldehyde oxidase was immobilized by absorption onto Sepharose 4B substituted with *n*-octylamine, using a procedure analogous to that previously described[1,22], or by absorption onto DEAE Sepharose CL 6B. Aldehyde oxidase-containing protein (15 mg) was applied *per* gram of freeze-dried CNBr-activated Sepharose 4B or to 3.5 ml packed gel of Sepharose CL 6B in a total volume of 30 ml 0.03 M borate buffer, pH=9.0, containing 0.1 mM EDTA. Adsorption was accomplished by rotation of the mixture in a round-bottomed flask overnight at 4°C. After washing the material several times with the same buffer, the aldehyde oxidase preparation was packed into a column and again washed with an appropriate buffer. In the case of DEAE Sepharose CL 6B, 5mM Tris-phosphate buffer pH=7.8 (0.1 mM EDTA) was used.

Oxidation. A buffered substrate solution (1.5-2.5 mM) was recirculated through the column using a pump and the conversion of substrate was detected by measuring the formation of product at 292 nm. When complete conversion was required, several columns with fresh immobilized enzyme were employed until all the substrate was consumed. After termination of the reaction, the column was run dry, the effluent evaporated and the product dried weighed and analyzed.
3c: M^+, *m/e* 180; ^{1}H NMR (D_2O) δ 6.55 (H-5, d), 7.84 (H-4, dd), 8.20 (H-2, d); $J_{2,4}$= 2.7 Hz and $J_{4,5}$= 9.2 Hz.
The residues of the reactions with 1d and 1e were purified by column chromatography eluting with chloroform/acetone (1:1). The solvent was evaporated and the isolated products were redissolved in minimal amounts of methanol. After a second purification, utilizing preparative HPLC, the collected samples of respective products were combined, the solvent evaporated and the products dried and

characterized.
3d: GC-MS: M^+, *m/e*= 180. Exact mass measurement gave for $C_9H_{12}N_2O_2$ (M^+): 180.0902 (theoretical: 180.0899). UV (H_2O) λ_{max} 261 (log ε 4.05), 298 (0.58); UV (1N HCl) λ_{max}: 261 (4.00).
4d: GC-MS: M^+, *m/e*= 180. Exact mass measurement gave for $C_9H_{12}N_2O_2$(M^+) 180.0905 (theoretical: 180.0899). UV (H_2O) λ_{max}: 259 (log ε 4.04), 282 (3.63); UV (1N HCl) λ_{max}: 239 (3.90).
4e: GC-MS: M^+, *m/e*= 194. Exact mass measurement gave for $C_{10}H_{14}N_2O_2$(M^+): 194.1039 (theoretical: 194.1055). UV (H_2O) λ_{max}: 258 (log ε 4.03), 281 (3.63), UV (1N HCl) λ_{max}: 240 (3.89).

Kinetic assays with aldehyde oxidase

The method used was a slight modification of the procedure described by Felsted *et al.*[9]. The assay mixture contained oxygen as final electron acceptor. 0.1 mM sodium EDTA, Tris-HCl buffer (ionic strength I=0.01) pH=9.0 and the substrate to be oxidized at appropriate concentrations in a final volume of 2.5 ml. Each assay (performed in duplicate) was initiated by addition of 0.1 ml of enzyme solution (approx. 1.5/mg/ml in Tris-HCl (I=0.01, pH=9.0)). The temperature of the assay mixture was maintained at 25°C. The oxidation of substrates was followed at 292 nm using an Aminco DW-2a spectrophotometer operating in split-beam mode. The rate was determined from the initial slope of the absorbance *versus* time, representing the rate of the formation of product. The molar differential absorption coefficients (log ε) at this wavelength are: 1a-3a, 3.64; 1b-3b, 3.68; 1c-3c, 3.67.
The inhibition of 1a oxidation by its homologues was determined under the same assay conditions as described above. Kinetic data and inhibition constants were calculated from Lineweaver-Burk[12] plots and replots, respectively.

Acknowledgements

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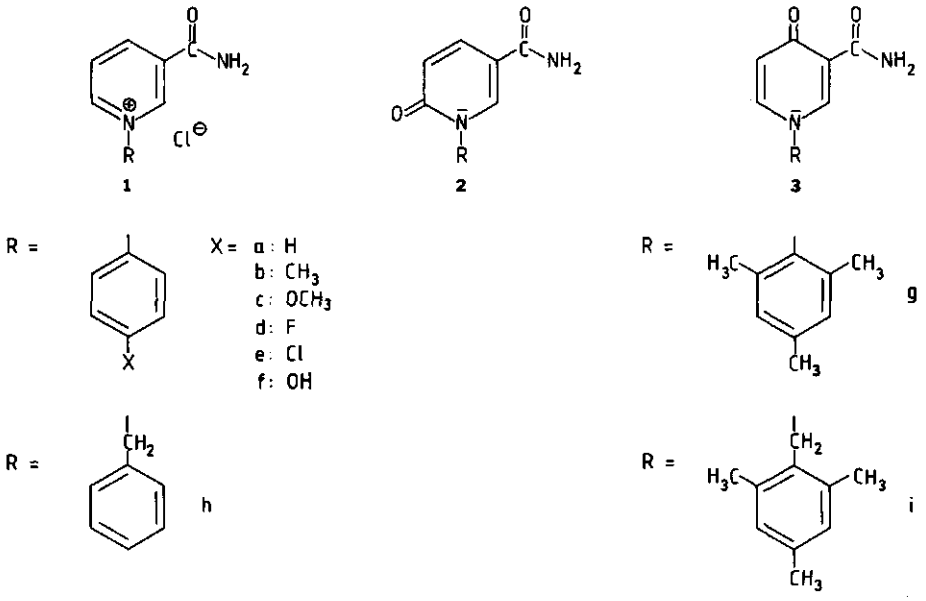
3 THE OXIDATION OF 3-AMINOCARBONYL-1-ARYL-PYRIDINIUM CHLORIDES BY RABBIT LIVER ALDEHYDE OXIDASE AND BOVINE MILK XANTHINE OXIDASE

3.1 INTRODUCTION

In a recent publication[1] we described the oxidation of 1-alkyl-3-aminocarbonyl-pyridinium chlorides (1, R = alkyl) by the enzyme aldehyde oxidase[2] derived from rabbit liver. Depending upon the size of the 1-alkyl substituent, 1-alkyl-1,6-dihydro-6-oxo-3-pyridinecarboxamide (2, R = alkyl) and/or its 4-oxo isomer (3, R = alkyl) were formed; the formation of product shifted from the 6-oxo to the 4-oxo compounds on increasing the size of the alkyl substituent. The oxidation of 3-aminocarbonyl-1-methylpyridinium chloride (1, R = methyl) by bovine milk xanthine oxidase and by aldehyde oxidase, both of which are molybdenum iron-sulphur flavin hydroxylases[2], only occurred at the C-6 position[1,3]. In contrast, oxidation of 1-arylpyridinium salts with xanthine oxidase yielded only the corresponding 4-oxo compounds[3]. We therefore decided to investigate the oxidation of 3-aminocarbonyl-1-arylpyridinium chlorides 1 (Scheme 3.1) with both enzymes.



Scheme 3.1

These compounds contain large substituents on the ring nitrogen atom and our aim was to see whether the steric effect of the substituent would direct the oxidation (in particular by aldehyde oxidase) to position 4 of the pyridinium ring. In addition, the effects of *para* substituents in the aryl group on the oxidation rates of aldehyde oxidase and xanthine oxidase were studied. The preparation of 4-oxo and 6-oxo products of several 3-aminocarbonyl-1-aryl-pyridinium chlorides using immobilized aldehyde oxidase, as well as both free and immobilized xanthine oxidase, are also described.

3.2 RESULTS AND DISCUSSION

3.2.1 Product analysis

HPLC analysis of the reaction mixtures, obtained by incubation of the 3-aminocarbonyl-1-arylpyridinium chlorides (1a-f) with aldehyde oxidase, showed the formation of two products with one product being preferentially formed.
In contrast, the substrates 1g-i gave only one product; for 1h the product was identified as 1-benzyl-1,6-dihydro-6-oxo-3-pyridinecarboxamide (2h) by comparison with an authentic sample. Two products are also formed on reaction of the substrates 1a-f with xanthine oxidase, corresponding - based on retention times - with the products formed by aldehyde oxidase. However, there is a significant difference between both enzymic reactions with respect to the ratio of the two products formed: the main product formed by aldehyde oxidase is the minor product in the xanthine oxidase reaction and *vice versa*. No oxo products are formed on oxidation of compounds 1g-i by xanthine oxidase.

3.2.2 Enzymic oxidation on a preparative scale

In order to establish the structure of the compounds obtained in the enzymic oxidations we performed reactionson a small preparative scale, using both free and immobilized enzyme, and isolated each of the products. The enzymic oxidation of compounds 1a-f with immobilized aldehyde oxidase was carried out at pH=7.0. The most favourable pH for xanthine oxidase ranges from about 9 to 11. Below pH=9.0,this enzyme hardly showed any activity towards compounds 1, as was also observed for some 1-arylpyridinium salts[4]. Above pH=11.2, rapid inactivation of xanthine oxidase occurred and at these pH values the pyridinium salts are subject to amide hydrolysis and nucleophilic attack by hydroxyl ions[5,6]. The experiments with this enzyme were therefore carried out at about pH=10. At this pH, the rate of oxidation is sufficiently fast and no chemical degradation of the substrates is found in solutions containing no enzyme (UV detection[6]).

A. Oxidation of compounds 1a-i with immobilized aldehyde oxidase

Table 3.5 summarizes the yields and analytical data of the 6-oxo (2) and 4-oxo products (3), obtained in the reactions with immobilized aldehyde oxidase. Oxidation of substrates 1a, 1b and 1d-f afforded mainly the 6-oxo products (2) together with a minor amount of the corresponding 4-oxo compounds (3). Exceptions were 1c, which gave a higher yield of the 4-oxo analogue than did the other compounds, and 1g, which gave exclusively the 4-oxo compound. Rabbit liver xanthine oxidase, which is also present in the enzyme preparation used[7], does not contribute to the oxidation of the substrates since isolated rabbit liver xanthine oxidase showed no activity under the experimental conditions employed. The yields of the enzymic oxidations vary from 12 to 88% (Table 3.5). The relatively low yields of 1e, 1h and 1i are possibly due to the formation of by-products during the reaction. To what extent these "by-products" originate from other minor enzymic activities present in the enzyme preparation and/or have been caused by inadequacy of the aldehyde oxidase reaction is not clear.

B. Oxidation of compounds 1a-f with free and immobilized xanthine oxidase

Oxidation of the substrates 1a-f with free xanthine oxidase at pH=9.8 gave mainly the 4-oxo products; the yields of isolated product and the analytical data of the compounds obtained are given in Table 3.6. In all the reactions small amounts of 6-oxo products (< 5%) were formed. Oxidation of substrate 1a with xanthine oxidase at pH=9.1 , rather than at pH=9.8, did not improve the yield of 3a. When an aqueous solution of substrates 1a or 1b (pH=10.2) is passed through a column packed with *immobilized* xanthine oxidase[8], product yields of 27 and 18% (Table 3.6) are obtained. With this enzyme the formation of "by-products" is also observed.

3.2.3 Structure assignment of the oxidation products

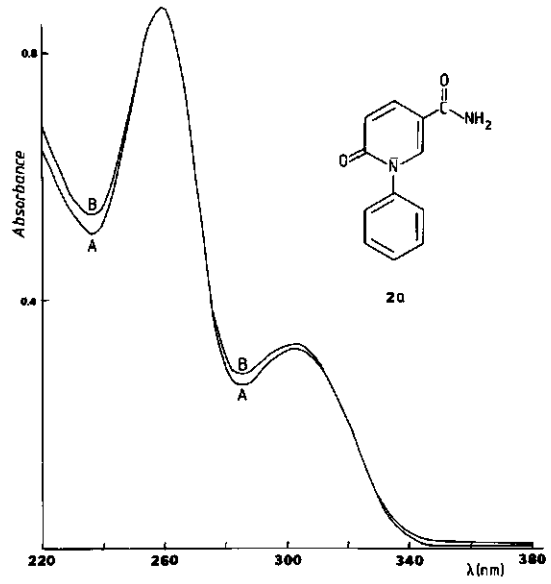
The structure assignment of the oxidation products is based upon ^{1}H NMR data (Table 3.1) and upon UV spectroscopy (Figure 3.2). The *ortho* coupling constants of the pyridine ring protons in the ^{1}H NMR spectra are of diagnostic value in the assignment of the position of the oxo group in the ring[1]. The coupling constant is 9.6 Hz for all 6-oxo compounds and 7.5 Hz for the corresponding 4-oxo isomers. This is in agreement with the values determined for the related 1-alkyl-1,6-dihydro-6-oxo-3-pyridinecarboxamides (2, R = alkyl) and their 4-oxo analogues (3, R = alkyl)[1].

Table 3.1 Chemical shifts of the pyridine ring protons of the oxidation products in CD_3OD[a]

Compound	H_2	H_4	H_5	H_6
2a	8.27	8.01	6.60	
3a	8.80		6.72	8.10
2b	8.24	8.03	6.65	
3b	8.67		6.62	8.00
2c	8.24	8.00	6.60	
3c	8.60		6.60	7.93
2d	8.26	8.01	6.60	
3d	8.70		6.65	8.02
2e	8.25	8.00	6.60	
3e	8.76		6.69	8.08
2f[b]	8.22	7.85	6.40	
3f[b]	8.43		6.48	8.07
3g	8.38		6.69	7.72
2h	8.37	7.92	6.55	
2i	7.66	7.89	6.57	

a. For compounds 2: $J_{2,4}$= 2.5 Hz, $J_{4,5}$= 9.6 Hz; compounds 3: $J_{2,6}$= 2.4 Hz; $J_{5,6}$= 7.5 Hz.
b. In DMSO-d_6.

Another criterion is the chemical shift value of H_2, which is at lower field in the 4-oxo compounds than in the corresponding 6-oxo compounds[1]. It is of interest to note that the chemical shift of H_2 in compound 2i exhibits an upfield shift of about 0.7 ppm in comparison to H_2 in 2h. Calculation of $\Delta\delta(H_2\text{-}H_5)$ and $\Delta\delta(H_2\text{-}H_4)$ for the compounds 2h and 2i gives 1.82 and 0.45 ppm for 2h and 1.09 and -0.23 ppm for 2i, yielding a net difference between both compounds of 0.73 and 0.68 ppm, respectively. This upfield shift may be rationalized by assuming a strong shielding effect of the 2,4,6-trimethylbenzyl group. Two extreme conformations, in which both ring planes A en B face each other (I) or are at right angles (II), are shown in Figure 3.1[9a,b]. These conformations are transformed into one another by a rotation of ring B 90° about the C-N bond. As a consequence of steric hindrance it is to be expected that, especially for



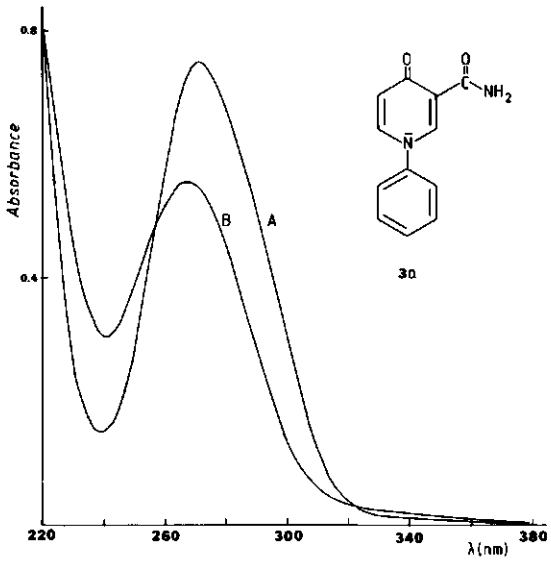


Figure 3.2 UV spectra of the enzymic oxidation products 2a (0.060 mM) and 3a (0.037 mM) in water (A) and 1N HCl (B).

R = CH_3, conformation II will be more favourable than I, leading to the shielding effect of H_2 of the pyridine ring by the 2,4,6-trimethylbenzyl group.

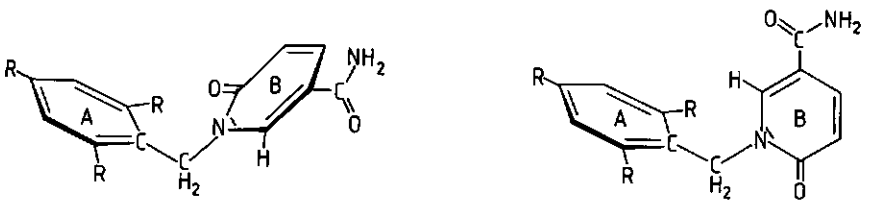


Figure 3.1 Two conformations of compounds 2h and 2i resulting from rotation about the C-N bond.

The UV spectra of the corresponding 4-oxo and 6-oxo products are different in the range 220-360 nm (Figure 3.2) and therefore are of diagnostic value. The 6-oxo products obtained here showed hardly any spectral change and the 4-oxo compounds altered only to a minor extent when the spectra were recorded in 1 N hydrochloric acid instead of water. This is in contrast to the large effect found with 1-alkyl-1,4-dihydro-4-oxo-3-pyridinecarboxamides (3, R = alkyl)[1]. The spectral data of the products are summarized in Tables 3.5 and 3.6.

3.2.4 Influence of aryl substituents on the rate of oxidation with aldehyde oxidase

Kinetic data obtained with free aldehyde oxidase at pH=9.8 are collected in Table 3.2. The logarithmic plot of the relative maximal oxidation rates at the C-6 position by aldehyde oxidase *versus* the substituent constant σ of X is shown in Figure 3.3 for substrates 1a-e.

A reaction constant ρ of approximately 3.6 can be calculated from the slope of the line. This large positive reaction constant indicates that the rate-limiting step in the reaction is facilitated by a low electron density at the reaction site and is very sensitive to substituent effects. When we assume a mechanism of oxidation by aldehyde oxidase, analogous to that proposed for milk xanthine oxidase[10], on the basis that the two enzymes are structurally closely related[11,12],

then our data strongly indicate that nucleophilic attack by a persulfide group[10] or a terminal oxygen ligand of molybdenum[13] at the catalytic site of the enzyme significantly influences the rate of oxidation.

The nature of the nucleophilic species at the active site is comparable with that of a methoxide ion, since for the addition of these ions to 1-(3- or 4-substituted phenyl)pyridinium salts at C-6 (or C-2) a ρ-value of 3.55 was found[14].

Table 3.2 Maximum rates for the oxidation of 3-aminocarbonyl-1-arylpyridinium chlorides by free aldehyde oxidase and xanthine oxidase at pH=9.8

Substrate	Aldehyde oxidase		Xanthine oxidase	
	$V_{6\text{-oxo}}$[a]	$V_{4\text{-oxo}}$[a,b]	$V_{6\text{-oxo}}$[a,c]	$V_{4\text{-oxo}}$[a,c,d]
1a	0.43 ± 0.02	-		0.41 ± 0.02
1b	0.151 ± 0.009	-		0.30 ± 0.01
1c	0.058 ± 0.008	0.023 ± 0.003		0.34 ± 0.01
1d	1.46 ± 0.23	-		0.39 ± 0.02
1e	2.92 ± 0.53	-		0.32 ± 0.01
1g		0.56 ± 0.03		
1h	0.050 ± 0.003			
1i	0.133 ± 0.007			
AMP[e]	0.48[f] ± 0.02		0.34 ± 0.01	
			0.62 ± 0.03[g]	

a. In µmol/min mg.

b. No accurate data could be determined for substrates 1a, 1b, 1d and 1e.

c. Ferricyanide was used as terminal electron acceptor.

d. The small contributions of the oxidation to 6-oxo product were neglected (< 5%).

e. AMP = 3-aminocarbonyl-1-methylpyridinium chloride.

f. K_M = 0.131 ± 0.005 mM.

g. This value was obtained using O_2 as final electron acceptor; K_M = 16.9 ± 0.7 mM.

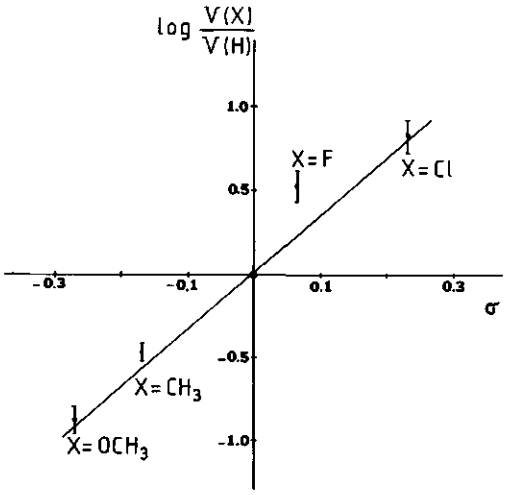


Figure 3.3 Hammett plot for the oxidation at the C-6 position of 3-aminocarbonyl-1-arylpyridinium chlorides. Oxidation by free aldehyde oxidase at pH=9.8

It is remarkable that only in the case of the *p*-methoxy substituent it was also possible to measure the maximum rate of formation of the 4-oxo product . The rate was found to be about 2.5 times lower than that for oxidation at C-6. However, it is unlikely that the increase in the rate of oxidation at C-4 arises from the electron-donating character of the *para* substituent in the phenyl ring, since incubation of substrate 1f, containing a strong electron-donating hydroxy substituent, showed no increased formation of the 4-oxo product as compared to that of the substrates 1a-e (HPLC analysis). At pH=9.8 the hydroxy group in this compound (pK_a = 9.25 ± 0.05) is to a large extent ionized, which increases the electron-donating properties even more. Therefore, the rates of oxidation at C-4 and C-6 with substrate 1f were very low at this pH and no accurate rate data could be obtained. Testing of substrate 1a in the pH-range 6.4 to 10.2 gave, after correction for buffer effects, a constant maximal oxidation rate in the pH range 7.5-10.2. At values below pH=7.5, a gradual decrease in reaction rate was observed. At pH=6.4, the maximal rate was about 75% of the value determined between pH=7.5-10.2. The oxidaton at the C-4 position of substrate 1g by aldehyde oxidase occurred at a rate about 24 times higher than that at C-4 in 1c and much higher than for substrates 1a,1b,1d-f, for which no kinetic data for the oxidation at C-4 could be obtained. This effect may be ascribed

to a strong preference of 1g for a conformation in which both aromatic rings are out of plane. We assume that this conformation will be partially retained in the enzyme-substrate complex since it is energetically most favourable. As a consequence, the orientation of the 2,4,6-trimethylphenyl group in the hydrophobic pocket of the enzyme[1] may force the nucleophilic species[10,13] in such a position that only attack at C-4 is possible.

To confirm whether or not oxidation at both the C-4 and C-6 positions originates from catalysis by aldehyde oxidase we carried out inhibition experiments using menadione (2-methyl-1,4-naphthoquinone), which is a very potent inhibitor of aldehyde oxidase[7,15]. With a menadione concentration of 10 μM, and substrate concentrations for which almost maximum oxidation rates had been found, the activity for the substrates 1a and 1g, for instance, completely disappeared.

The maximal oxidation rate at the C-6 position of substrate 1h is rather low in comparison to the rate of oxidation for compound 1a: compared to the oxidation of 3-aminocarbonyl-1-methylpyridinium chloride (1, R = methyl), the rate is only 10%. As previously noted for a number of alkyl substituents[1], this effect is probably due to steric hindrance, caused by the N-1 substituent, towards nucleophilic attack. The effect is not present or masked for compounds 1a-f by the overriding electronic effects on the oxidation site. In this way the fact that the oxidation rates for 1h and 1i differ by a factor of 2.5 can be explained by the disparity in accessibility of the C-6 position in both substrates.

Table 3.3 Chemical shifts of the pyridinium ring protons of compounds 1h and 1i in D_2O

Hydrogen	Compounds		
	1h	1i	$\Delta\delta$[a]
H_2	9.52	9.09	
H_4	9.07	8.93	
H_5	8.39	8.19	
H_6	9.29	8.80	
$H_2 - H_4$	0.45	0.16	0.29
$H_2 - H_5$	1.13	0.90	0.23
$H_6 - H_4$	0.22	-0.13	0.35
$H_6 - H_5$	0.90	0.61	0.29

a. Upfield shifts relative to 1h.

This phenomenon is illustrated by the ^{1}H NMR data (Table 3.3), which show upfield shifts for the H_2 and H_6 protons in the pyridinium ring of compound 1i compared to 1h. The upfield shifts are in agreement with data which have been found by Verhoeven *et al.*[9a] for 1-benzylpyridinium compounds ($\Delta\delta$=0.25 ppm). The explanation of the effect is analogous to that given for the oxidation products 2h and 2i. Accordingly, it is concluded that the steric conformation of substrate 1i tends to approximate structure II (Figure 3.1), which we presume to be partially retained in the enzyme-substrate complex. Thus, in 1i, C-6 is more accessible towards nucleophilic attack than it is in compound 1h. Compared to 1g, the interaction of the aryl substituents of 1h and 1i with the hydrophobic site of the enzyme[1] results in oxidation at C-6 because of the different stereochemical distortion induced by the methylene group. These results support our assumption that steric factors control the position of nucleophilic attack in the reaction pathway of the oxidation of these compounds by aldehyde oxidase.

3.2.5 Influence of aryl substituents on the rate of oxidation with xanthine oxidase

In the kinetic assays using free xanthine oxidase the oxidation rate was determined by following the reduction of ferricyanide. The assay of product formation was less feasible, since the high substrate concentrations (0.3-4 mM) required at pH=9.8 caused high UV absorptions at potential assay wavelengths.
The influence of substituent X on the maximum oxidation rate at C-4 by this enzyme is rather small (Table 3.2). As a single effect the rate of oxidation shows a moderate decrease when a substituent larger than hydrogen is introduced. With substrate 1f, no maximal rate could be determined because of the interference of ferricyanide reduction (*i.e.* decolouration) and the yellow colour of the substrate in solution at pH=9.8. Compared to the oxidation of 3-aminocarbonyl-1-methylpyridinium chloride (1, R = methyl), the substrates 1a-e are oxidized at a similar rate by xanthine oxidase. It is obvious that both enzymes, in spite of their close structural relationship, exhibit a different behaviour towards the substrates 1 with respect to the oxidation site. This is also reflected in the different affinity of both enzymes towards 3-aminocarbonyl-1-methylpyridinium chloride (1, R = methyl). For aldehyde oxidase and xanthine oxidase, Michaelis constants (K_M) of 0.131 ± 0.005 mM and 16.9 ± 0.7 mM were found, respectively.

3.3 EXPERIMENTAL SECTION

Melting points are uncorrected. Mass spectra were determined using an AEI MS-902 mass spectrometer, equipped with a VG ZAB console. The ^{1}H NMR spectra were recorded on a Hitachi Perkin Elmer R-24B, a Varian EM-390 or a Bruker CXP-300 spectrometer, with TMS or DSS as internal standard (δ=0 ppm). UV spectra were recorded on an Aminco DW-2a UV-VIS spectrophotometer. HPLC analyses were performed using a Varian 5000 instrument equipped with a Micro Pak MCH-10 column (30x0.4 cm), a Schoeffel GM-770 monochromator and an SF-770 spectroflowmonitor. Operating conditions were Δp= 17.6 MPa, eluent water/methanol 50/50 (v/v), flow rate 2.0 ml/min. The ionization constant of 3-aminocarbonyl-1-(*p*-hydroxyphenyl)pyridinium chloride was determined by titration. Column chromatography was carried out over Merck Silica gel 60 (70-230 mesh ASTM). Bovine milk xanthine oxidase (E.C.1.2.3.2) was purchased from Boehringer. Partially purified aldehyde oxidase (E.C.1.2.3.1) was prepared from frozen rabbit livers as previously described[1]. This type of preparation was used for kinetic assays. A less purified enzyme preparation, acquired by ommission of the hydroxylapatite step in the purification procedure, was employed for use in organic synthesis.
Rabbit liver xanthine oxidase (E.C.1.2.3.2) was isolated according to the same procedure as used for aldehyde oxidase, except that xanthine oxidase was eluted from the hydroxylapatite column by a linear gradient of 200-400 mM potassium phosphate buffer, pH=7.8 containing 0.1 mM EDTA. Only fractions free of detectable aldehyde oxidase activity were pooled.

Preparation of starting materials

1-Benzyl- (1h) and 1-methyl-3-aminocarbonylpyridinium bromide or iodide[5] were converted into the corresponding chlorides by passage over a Dowex 1-X2 column. Menadione (98%) was obtained from Aldrich and purified by recrystallization from ether.

3-Aminocarbonyl-1-arylpyridinium chlorides (1a-g)

3-Aminocarbonyl-1-(2,4-dinitrophenyl)pyridinium chloride[16] (3.25 g, 10 mmol) was dissolved in 15 ml of methanol and an equimolar amount of the appropriate arylamine was added with stirring. The colour of the solution immediately turned dark-red, but soon became orange-red and a precipitate was formed. If necessary, the reaction was completed by refluxing the mixture for 30-60 min. After cooling, the precipitate (2,4-dinitroaniline) was filtered off and the filtrate diluted with ether. The precipitate thus formed was filtered and recrystallized twice

Table 3.4 Analytical data of 3-aminocarbonyl-1-arylpyridinium chlorides

Compound	Yield (%)	M.p.(°C)	Experimental		Theoretical		$\lambda_{max}^{H_2O}$(nm)	log ε
			%C	%H	%C	%H		
1a	50	254-255[c]	61.70	4.62	61.41	4.72	272	3.88
1b	45	268-270[d]	62.85	5.45	62.78	5.27	281	3.76
1c	85-90	270-272	59.27	5.12	58.98	4.95	245;317	3.85;3.79
1d	80	288-290	57.23	4.29	57.04	3.99	274	3.89
1e	60	301-303	53.58	3.84	53.55	3.75	277	3.91
1f[a]	45	257-260	56.77	4.49	57.49	4.42	246;318	6.64;4.81
1g[b]	50	288-290	61.38	6.39	61.11	6.50	264	3.67
1i	85-90	240-241	65.94	6.44	66.08	6.59	265	3.64

a. Exact mass measurement for dihydro compound $C_{12}H_{12}N_2O_2$[17] (M^+): exp. 216.0886; theor. 216.0899.

b. Contained 1 mol of water.

c. Lit.[18]: 238-239°C.

d. Lit.[18]: 209°C.

from absolute ethanol or acetone. The yields and analytical data of the products are summarized in Table 3.4.

3-Aminocarbonyl-1-(2,4,6-trimethylbenzyl)pyridinium chloride (1i)

Nicotinamide (1.22 g, 10 mmol) was dissolved in tetrahydrofuran (25 ml) and 2.08 g (11 mmol) 2,4,6-trimethylbenzyl chloride was added. The mixture was refluxed and stirred for a few hours. The precipitate thus obtained was filtered (1.45 g) and recrystallized twice from acetone. Analytical data are given in Table 3.4.

1-Benzyl-1,6-dihydro-6-oxo-3-pyridinecarboxamide (2h)

This compound was acquired in a small yield by oxidation of 1h with alkaline ferricyanide according to the procedure of Möhrle and Weber[19]. The crude product was purified by column chromatography with chloroform/methanol (30/1) as eluent, m.p. 172-173°C (lit.[20] 173-173.5°C).

HPLC analysis

The HPLC analysis of reaction mixtures was performed as described previously[1]. The enzymatic reactions with xanthine oxidase were carried out in a borate buffer, pH=9.8 (I=0.05; 0.1 mM EDTA). Registration of the oxidation products occurred by UV detection at 254 nm (log ε for 2a: 4.14; 3a: 4.07; 2b: 4.20; 3b: 3.95; 2c: 4.10; 3c: 4.00; 2d: 4.13; 3d: 3.98; 2e: 4.12; 3e: 3.86; 2f: 4.12; 3f: 3.89; 3g: 4.15; 2h: 4.12; 2i: 4.09).

Enzymic synthesis of products

A. Aldehyde oxidase was immobilized by adsorption onto DEAE Sepharose CL 6B as previously described[1], with the exception that the quantity of protein applied during immobilization was increased three- to fourfold. The immobilized enzyme preparation was packed in a column and washed with 10 mM potassium phosphate buffer, pH=7.0 (0.1 mM EDTA) at 4°C. It was established that the yield of products was maximal when the reaction was performed at neutral or slightly acidic pH, *i.e.* pH=6.4-7.0. The amount of aldehyde oxidase used per column for each conversion was 12 units. (for definition see *Kinetic assays*). After depletion of the aldehyde oxidase activity, the ion exchanger was regenerated as described elsewhere[21].

300 ml of a 0.5 mM substrate solution (in the same buffer) was slowly (0.25 ml/min) passed through the column at 4°C and the conversion of substrate detected by registration of the formation of product(s) at 254 nm (HPLC). In the case of a rather slow reaction, the solution was recirculated using a pump. The collected effluent was evaporated to dryness and the residue purified by column

Table 3.5 Analytical data of 1-aryl-1,4-dihydro-4-oxo- and 1-aryl-1,6-dihydro-6-oxo-3-pyridinecarboxamides obtained by oxidation using immobilized aldehyde oxidase

Sub-strate	Product	Yield (%)	M.p.(°C)	Experimental Exact mass	%C	%H	Theoretical Exact mass	%C	%H	$\lambda_{max}^{H_2O}$(nm)(log ε)	λ_{max}^{1NHCl}(nm)(log ε)
1a	2a	81	231-233	214.0739	67.54	4.67	214.0742	67.28	4.70	259(4.16);303(3.72)	259(4.15);302(3.73)
	3a[a]	< 5									
1b	2b	65	245-247	228.0894	68.12	5.02	228.0899	68.40	5.30	258(4.21);304(3.76)	258(4.20);303(3.76)
	3b[a]	10		228.0903							
1c	2c	52	233-234	244.0846	64.08	4.82	244.0848	63.92	4.95	258(4.10);305(3.66)	258(4.10);305(3.68)
	3c[a]	34		244.0846							
1d	2d	75	262-263	232.0655	62.20	3.86	232.0648	62.07	3.91	259(4.15);304(3.70)	259(4.14);303(3.69)
	3d[a]	< 5									
1e	2e	42	217.5-219	248.0350	57.48	3.86	248.0353	57.96	3.65	258(4.13);304(3.69)	258(4.13);304(3.70)
	3e[a]	< 5									
1f	2f	76	>315[d]	230.0695	62.90	4.50	230.0691	62.60	4.38	259(4.14);305(3.64)	259(4.14);305(3.67)
	3f[a]	11		230.0694							
1g	3g[b]	88	173-174	256.1212	61.82	6.80	256.1212	61.63	6.90	262(4.20)	250(3.99)
1h	2h[c]	57	172-173	228.0900			228.0899			260(4.13);298(3.73)	260(4.11);296(3.73)
1i	2i[c]	12	130-132	270.1377			270.1368			261(4.12);300(3.74)	260(4.10);296(3.74)

a. For analytical data, see Table 3.6.

b. Microanalytical data based on 2 mol of water.

c. Only exact mass measurements are given. From compound 2i insufficient material was obtained after recrystallization to allow microanalysis. Compound 2h was obtained by chemical oxidation.

d. Decomposition.

Table 3.6 Analytical data of 1-aryl-1,4-dihydro-4-oxo-3-pyridinecarboxamides obtained by using free xanthine oxidase

Sub-strate	Product	Yield (%)	M.p.(°C)	Experimental			Theoretical			$\lambda_{max}^{H_2O}$(nm)(log ε)	λ_{max}^{1NHCl}(nm)(log ε)
				Exact mass	%C	%H	Exact mass	%C	%H		
1a	2a[a]	< 5									
	3a	63;27[d]	221-222	214.0743	67.03	4.99	214.0742	67.28	4.70	271(4.31)	267(4.20)
1b	2b[a]	< 5									
	3b[b]	36;18[d]	189-190	228.0895	63.83	5.53	228.0899	63.40	5.73	273(4.24)	272(4.08)
1c	2c[a]	< 5									
	3c	42	205.5-207	244.0844	64.23	4.85	244.0848	63.92	4.95	276(4.30)	282(4.14)
1d	2d[a]	< 5									
	3d	59	247-248	232.0653	62.09	3.76	232.0648	62.07	3.91	270(4.22)	268(4.09)
1e	2e[a]	< 5									
	3e[c]	7	239-241	248.0354			248.0353			273(4.18)	271(4.06)
1f	2f[a]	< 5									
	3f	28	>315[e]	230.0693	62.31	4.27	230.0691	62.60	4.38	275(4.17)	282(3.97)

a. For analytical data, see Table 3.5.

b. Microanalytical data based on 1 mol of water.

c. From compound 3e, insufficient material was obtained to allow microanalysis.

d. Isolated from the reaction with immobilized enzyme.

e. Decomposition.

chromatography (chloroform/methanol 19:1) to separate both oxidation products and coloured by-products. After evaporation of the solvent, the crude product(s) was(were) weighed and recrystallized two or three times from distilled water. The yields of the crude products and the analytical data of the recrystallized products are given in Table 3.5.

B. Xanthine oxidase was used in free and immobilized state. A xanthine oxidase solution (2.5 ml containing 1 mg protein per ml) in borate buffer, pH=9.8 (I=0.05; 0.1 mM EDTA) was added to 15 ml of a 2 mM substrate solution in the same buffer and the mixture carefully stirred at 4°C. After 8 h, a further 2.5 ml of the enzyme solution was added and the reaction allowed to continue overnight. However, a much longer reaction time (∿72 h) was required for compound 1f, and consequently the enzyme preparation was added in aliquots of 0.5 ml at intervals during this period. After completion of the reaction, the mixture was brought to about pH 6.5 and evaporated to dryness. The work-up procedure of the residue was identical to that described under *A*. Five incubation mixtures were started at the same time to obtain enough product. Xanthine oxidase was immobilized according to the method of Tramper *et al.*[8]. For each conversion, 500 ml of fresh whole milk (obtained from the University farm) was used. The immobilized enzyme preparation was packed into a column and thoroughly washed with carbonate buffer, pH=10.2 (I=0.05;0.1 mM EDTA) at 4°C. Fifty ml of a 5 mM substrate solution (in the same buffer) was slowly (0.25 ml/min) recirculated through the column at 4°C and the conversion of substrate monitored using HPLC. When the reaction was completed, the column was run dry, the effluent acidified to about pH=6.5 and evaporated to dryness. Product isolation was carried out as described under *A*. The yields and analytical data are summarized in Table 3.6.

Kinetic assays

The assay for aldehyde oxidase was performed as previously described[1]. Each assay was carried out at least in duplicate and, with substrates 1d and 1e, in quadruplicate. Sodium borate, pH=9.8 with an ionic strength I=0.05, containing 0.1 mM EDTA (tetrasodium ethylenediamine tetraacetate), was used as buffer. Buffers of the same ionic strength [22] were employed for assays in the pH range 6.4-10.2. In the case where the formation of two products from one single substrate was followed, the initial reaction rate was monitored at two suitable wavelengths. The appropriate wavelengths (λ in nm) and corresponding molar differential absorption coefficients (log $\Delta\varepsilon$) at pH 9.8 are: 1a-2a: 318 (3.46),

1b-2b: 254 (4.08), 270 (3.84); 1b-3b: 254 (3.71), 270 (4.11); 1c-2c: 254 (3.82), 288 (3.60); 1c-3c: 254 (-), 288 (4.10); 1d-2d: 316 (3.48); 1e-2e: 316 (3.46); 1g-3g: 288 (3.73); 1h-2h: 3.06 (3.62); 1i-2i: 304 (3.72). These parameters are constant over the pH range studied, *i.e.* 6.4-10.2.

Substrate inhibition of free aldehyde oxidase with derivatives 1a-g at pH=9.8 was observed above 0.03-0.05 mM, depending upon the substrate employed. At lower pH, for example pH=7.0, the inhibition is observed at higher substrate concentrations (0.1-0.2 mM).

For aldehyde oxidase, one unit of enzyme activity is defined as the amount of enzyme which oxidized 1 µmole of 3-aminocarbonyl-1-methylpyridinium chloride *per* min at 25°C. The assay conditions were: 5 mM substrate in 50 mM potassium phosphate buffer, pH=7.8 (0.1 mM EDTA) with the reaction being monitored at 292 nm (log $\Delta\varepsilon$=3.64). Xanthine oxidase activity at pH=9.8 was determined under the same conditions as used for aldehyde oxidase, with potassium ferricyanide (0.4 mM) as final electron acceptor[3]. The reactions were followed spectrophotometrically by monitoring the change in absorbance at 420 nm (log $\Delta\varepsilon$=3.01). No substrate inhibition was established in the concentration range employed.

With 3-aminocarbonyl-1-methylpyridinium chloride as substrate, the xanthine oxidase activity was also measured at 292 nm using oxygen as electron acceptor.

All assays were performed in duplicate.

Kinetic data were calculated from Lineweaver-Burk plots[23].

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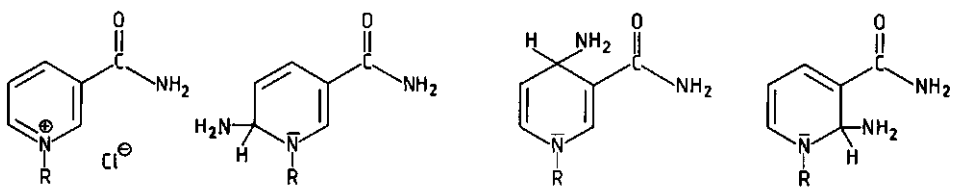
4 COVALENT AMINATION OF 1-ALKYL- AND 1-ARYL-3-AMINOCARBONYLPYRIDINIUM CHLORIDES AS "MODEL" FOR THE ENZYMIC ACTIVITY OF ALDEHYDE OXIDASE

4.1 INTRODUCTION

In previous papers the oxidation of 1-alkyl-(1a-e) and 1-aryl-3-aminocarbonyl-pyridinium chlorides (1f-h) by rabbit liver aldehyde oxidase into 1-alkyl(aryl)-dihydro-oxo-3-pyridinecarboxamides has been described[1,2](Scheme 4.1).
It was found that the nature of the substituent on the ring nitrogen (N-1) has a significant influence on the site of oxidation by the enzyme. In cases where the substituent at position 1 is a methyl , ethyl or *n*-propyl group the site of oxidation is exclusively position 6. If the N-1 substituent is *t*-butyl, only position 4 is oxidized[1]. This result indicates that the site of oxidation shifts from position 6 to position 4 with increasing size of the alkyl substituent. The aryl compounds show a somewhat different behaviour. They are oxidized predominantly at position 6, however some oxidation occurred at position 4, especially in 3-aminocarbonyl-1-(*p*-methoxyphenyl)pyridinium chloride (1g). Exclusive oxidation at position 4 was found with 3-aminocarbonyl-1-(2,4,6-trimethylphenyl)pyridinium chloride (1h)[2].



1 **2** **3** **4**

R= a: CH_3 , b: C_2H_5 , c: n-C_3H_7 , d: i-C_3H_7 , e: t-C_4H_9
f: C_6D_5 , g: 4-OCH_3-C_6H_4 , h: 2,4,6-tri-CH_3-C_6H_2

Scheme 4.1

The mechanism for the oxidation by aldehyde oxidase[2] is assumed to involve an initial nucleophilic attack by a persulfide group[3] or a terminal oxygen ligand of molybdenum[4] at the catalytic site of the enzyme, leading to an intermediary covalent σ-adduct. The position at which this adduct is formed determined the site where the oxo group is introduced in the substrate.
As a possible in-vitro model for covalent σ-adducts formed between compounds 1 and aldehyde oxidase, we considered the aminodihydro-3-pyridinecarboxamides, formed by reaction of 1 with liquid ammonia. In principle three σ-adducts *i.e.* the 6-amino-1,6-dihydro-(2), the 4-amino-1,4-dihydro-(3) and the 2-amino-1,2-dihydro-3-pyridinecarboxamides (4) can be obtained. It has already been reported[5a,b] that 3-aminocarbonyl-1-methylpyridinium chloride (1a) gives exclusively 2a in the temperature range -40 to 0°C. Replacement of the methyl substituent by a benzyl- or *p*-nitrobenzyl group does not change the addition pattern[5b]; with aliphatic amines exclusive addition at C-6 has also been observed for these compounds[6,7]. Addition of nucleophiles such as nitromethane[8], nitromethide ion[9], sulfite[8,10], methanethiolate[11], ethanethiolate[9], ethoxide[12], cyanide[8,12-14] or hydroxide ions[8,11,12,15] to 1 usually takes a somewhat different course, resulting in the formation of σ-adducts in which the nucleophile is attached to C-4 and/or to C-2 and C-6. The striking similarity between the site of nucleophilic addition in 1a with liquid ammonia and amines and the position of oxidation of 1a by aldehyde oxidase induced us to study the covalent amination of the pyridinium salts 1a-h in liquid ammonia in more detail.

4.2 RESULTS AND DISCUSSION

4.2.1 Covalent amination of 1-alkyl-3-aminocarbonylpyridinium chlorides

The reaction of compounds 1a-c with liquid ammonia gives rise to exclusive formation of the 6-amino-1,6-dihydro compounds 2a-c, as evidenced by ^{1}H NMR spectroscopy (Table 4.1). All proton signals are shifted upfield compared to the corresponding signals of 1a-c in D_2O. The shifts are most pronounced for the hydrogens attached to C-6 ($\Delta\delta$=4.32-4.51 ppm) due to the newly formed tetrahedral centre at C-6. The correct signal assignment is based on the chemical shift values and the coupling patterns and confirmed by the data, obtained by measurement of the ^{1}H NMR spectrum of 3-aminocarbonyl-*4-deuterio*-1-ethylpyridinium chloride in liquid ammonia. These data are in agreement with values published[5b].

Table 4.1 *^{1}H NMR data of the ring protons for 1-alkyl-6-amino-1,6-dihydro-3-pyridinecarboxamides 2 and 1-alkyl-4-amino-1,4-dihydro-3-pyridinecarboxamides 3 in liquid ammonia at -45°C*[a]

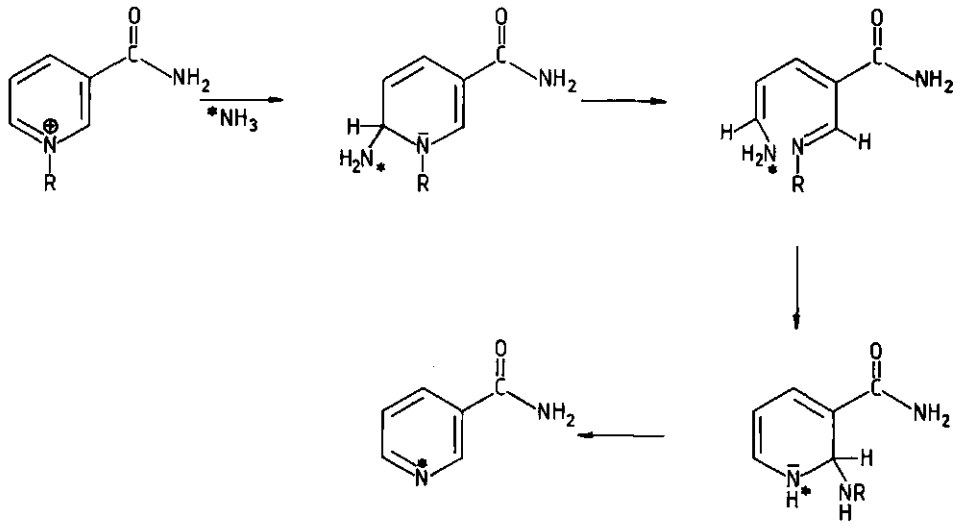
Compound	H-2	Δδ[b]	H-4	Δδ[b]	H-5	Δδ[b]	H-6	Δδ[b]
2a	7.29	1.97	6.55	2.35	5.06	3.14	4.66	4.32
2b	7.33	2.05	6.49	2.44	5.04	3.22	4.72	4.39
2c	7.32	2.15	6.54	2.51	5.07	3.31	4.71	4.51
2d	7.40	1.99	6.49	2.44	5.02	3.24	4.73	4.45
3d	7.16	2.23	4.15	4.78	c	-	6.20	2.98
2e	7.64	1.82	6.66	2.29	5.26	3.07	5.01	4.35
3e	7.47	1.99	4.26	4.69	c	-	6.52	2.84

a. Adduct 2: $J_{2,4}$= 1.5-1.8 Hz; $J_{2,6}$= 1.1 Hz; $J_{4,5}$= 9.1-9.8 Hz; $J_{5,6}$= 4.8-5.4 Hz; adduct 3: $J_{2,6}$= 2.0 Hz; $J_{4,5}$= 4.2 Hz; $J_{5,6}$ could not be determined due to overlap of signals.

b. Upfield shifts relative to the corresponding compounds 1 in D_2O.

c. Difficult to interpret due to overlap by the H-6 signal of the corresponding 6-adduct and to the low intensity for compound 3d.

Variation over a wide temperature range (-70 to 0°C) does not change the addition pattern. Prolonged exposure of for instance 1b to liquid ammonia at room temperature leads to dealkylation, yielding 3-pyridinecarboxamide[5a,b]. It can be excluded that the dealkylation takes place by a nucleophilic attack of ammonia on the N-ethyl group: reaction of 1b with ^{15}N-labelled ammonia (8.1% ^{15}N) resulted in a ^{15}N excess in the 3-pyridinecarboxamide (about 8%). This proves that during the dealkylation a degenerate ring transformation has taken place which involves addition of the nucleophile as first step, followed by ring opening and closure (ANRORC-mechanism)[16] as presented in Scheme 4.2.
The ^{1}H NMR spectra of compounds 1d-e in liquid ammonia are rather complex (Figure 4.1). It is concluded that *two* aminodihydro compounds are obtained from 1d-e, viz. the C-6 adducts 2d-e and in addition the C-4 adducts 3d-e (Table 4.1).
This is based on the chemical shifts of the C-6 adducts 2a-c, the upfield shift values (Δδ), the coupling constants and especially comparison with a more simple spectrum obtained from the reaction of 3-aminocarbonyl-1-*t*-butyl-*4-deuterio*-pyridinium salt with liquid ammonia.



Scheme 4.2

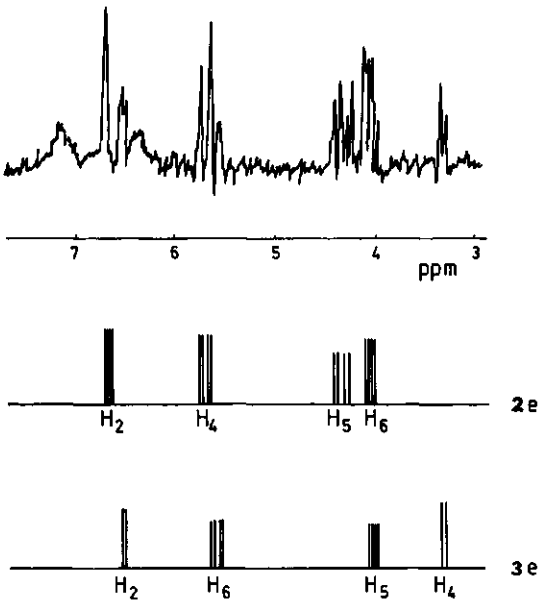
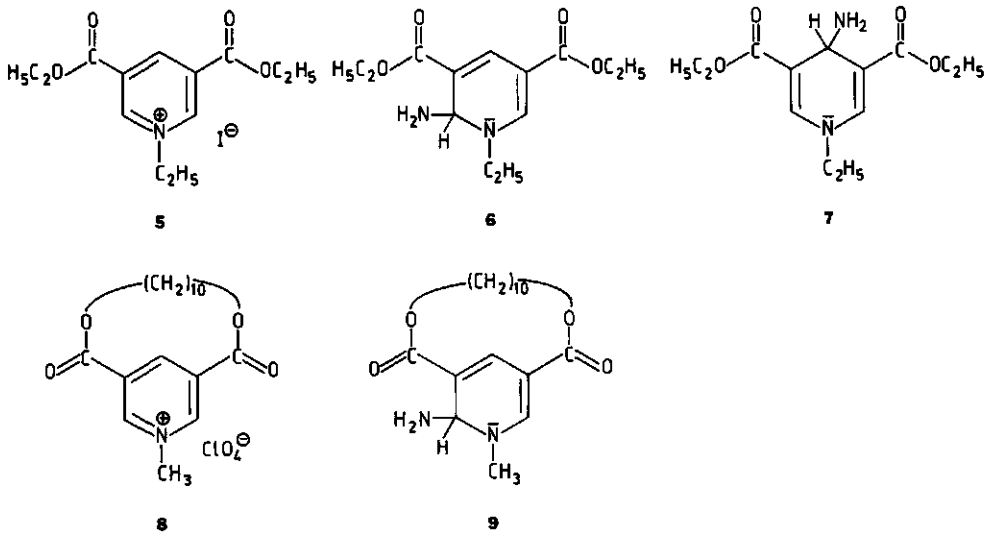


Figure 4.1 ^{1}H *NMR spectrum of 3-aminocarbonyl-1-t-butylpyridinium chloride 1e in liquid ammonia showing the signals and their assignments due to 6-adduct 2e and 4-adduct 3e.*

The ratio of 2d/3d is 9:1, the ratio of 2e/3e 6:4. These ratios are independent of the temperature in the range from -70° to 0°C. It is obvious from these results that the position of nucleophilic addition is not only dependent on the substituent at position 3, as was stated before[5a,b], but certainly on the nature of the substituent at position 1 as well. Moreover, it is evident that with an increasing size of the alkyl group at position 1, the addition at C-4 is promoted at the cost of addition at the adjacent C-6 position. Covalent amination in liquid ammonia is apparently rather susceptible to steric effects. This behaviour is also demonstrated by the addition pattern observed in the reaction of 3,5-dicarbethoxy-1-ethylpyridinium iodide (5) and its macrocyclic analogue 8 with liquid ammonia (Scheme 4.3). In compound 8 position 4 is less accessible for addition of nucleophiles because of steric interference. The ^{1}H NMR spectrum of 5 in liquid ammonia at -45°C shows the presence of two σ-adducts, viz. the C-6 (or C-2) adduct 6 and the C-4 adduct 7 (ratio 3:1). Adduct 6 is easily recognized by the appearance of three signals in the ratio 1:1:1, and adduct 7 by the presence of two signals with ratio 2:1 (Table 4.2).



Scheme 4.3

Table 4.2 Chemical shifts of the ring protons for compounds 5 and 8 and their σ-adducts in liquid ammonia at -45°C[a]

Compound	solvent	H-2	H-4	H-6
5	D_2O	9.73	9.47	9.73
6	NH_3	7.90	7.58	5.25
	Δδ	1.83	1.89	4.48
7	NH_3	7.49	4.55	7.49
	Δδ	2.24	4.92	2.24
8	DMSO-d_6	9.74	9.02	9.74
9	NH_3	7.81	7.60	5.14
	Δδ	1.93	1.42	4.60

a. Adducts 6 and 9: $J_{2,4}$ = 1.5 Hz; adduct 7: $J_{2,6}$ could not be determined.

At temperatures lower than -45°C, both adducts 6 and 7 are still present, but no accurate determination of the adduct ratio is possible due to limited solubility. At temperatures above -45°C the ^1H NMR spectrum changes: the signals attributed to 7 disappear and only the signals of adduct 6 remain. Apparently at low temperature a kinetically favoured process leading to 7 takes place; at higher temperature only the thermodynamically more stable adduct 6 is formed. Crown ester 8 in which position 4 is sterically hindered for nucleophilic addition gives exclusively the C-6 adduct in liquid ammonia at -45°C (Table 4.2). This result shows that position 4 in 8 is less accessible and that addition at C-6 is the most favoured process when no steric influence of the N-1 substituent is operative.

In Table 4.3 the qualitative data of the covalent amination patterns with 1a-e are compared with the oxidation patterns found for these compounds in reaction with aldehyde oxidase[1]. Evidently a good agreement exists between the position of addition by liquid ammonia and the nucleophilic species active in the aldehyde oxidase-mediated reaction. Both reactions are susceptible to steric interference of the substituent at position 1, the enzymic oxidation to a greater extent than the amination reaction. This is understandable because the nucleophilic species in the enzymic reaction is fixed in the catalytic site of the enzyme molecule and therefore has a greater steric interaction with the N-1 substituent.

Table 4.3 Comparison between the site of amination with liquid ammonia at -45°C and the site of oxidation by aldehyde oxidase of compounds 1a-h

Compound	oxidation	amination
1a	C-6	C-6
1b	C-6	C-6
1c	C-6	C-6
1d	C-6/C-4	C-6/C-4
1e	C-4	C-6/C-4
1f	C-6/C-4	C-6/C-2
1g	C-6/C-4	C-6/C-2
1h	C-4	C-6/C-2/C-4

In addition, the orientation of the substrate in the active site is of course sterically governed, leading to exclusive oxidation into a 4-oxo product in the case of the 1-*t*-butyl derivative 1e.

4.2.2 Covalent amination of 3-aminocarbonyl-1-arylpyridinium chlorides

The chemical shifts and coupling constants derived from the ^{1}H NMR spectra of 1-aryl compounds 1f and 1g in liquid ammonia (Table 4.4) show that two σ-adducts are obtained from both compounds, viz. the C-6 adducts 2f,g and the C-2 adducts 4f,g.

The adduct structures have been assigned based on our knowledge of the ^{1}H NMR data of C-6 adducts (2a-e), the upfield shifts, the magnitude of the coupling constants and especially the ^{1}H NMR spectra of the *4-deuterio* derivatives of 1f and 1g in liquid ammonia. The main difference between the covalent amino adducts obtained from the 1-alkyl- and 1-aryl compounds is that the upfield shift values ($\Delta\delta$) of the hydrogens attached to the tetrahedral centres in the adducts are significantly smaller for the 1-aryl compounds.

Compound 1h yields a more complicated ^{1}H NMR spectrum which arises from the presence of three σ-adducts; the C-6 adduct is formed in excess.

Complete assignment of signals could not be made because of the complexity of the spectrum. Additional proof for the formation of the C-6, C-4 and C-2 adducts has been obtained from ^{13}C NMR spectroscopy (Table 4.5).

Table 4.4 *^{1}H NMR data of the ring protons for 6-amino-1-aryl-1,6-dihydro-3-pyridinecarboxamides 2 and 2-amino-1-aryl-1,2-dihydro-3-pyridinecarboxamides 4 in liquid ammonia at -45°C*[a]

Compound	H-2	Δδ[b]	H-4	Δδ[b]	H-5	Δδ[b]	H-6	Δδ[b]
2f	7.65	1.94	6.71	2.48	5.52	3.00	5.19	4.19
4f	5.73	3.86	7.05	2.14	c	-	6.93	2.45
2g	7.55	1.97	6.70	2.46	5.45	3.03	5.14	4.19
4g	5.65	3.87	d	-	c	-	d	-

a. Adduct 2: $J_{2,4}$= 1.5 Hz; $J_{4,5}$= 9.0 Hz; $J_{5,6}$= 5.5 Hz; $J_{4,6}$= 1.8 Hz; $J_{2,6}\leqslant$1.0 Hz, adduct 4: $J_{2,6}$= 1.8 Hz; $J_{4,5}$= 6.0 Hz; $J_{5,6}$= 7.4 Hz; $J_{2,4}\leqslant$1.0 Hz; $J_{4,6}\leqslant$1.0 Hz.
b. Upfield shifts relative to the corresponding compounds 1 in D_2O.
c. Difficult to interpret due to overlap by the H-5 signal of the corresponding 6-adduct.
d. These signals lie under the phenyl multiplet.

Table 4.5 *^{13}C NMR data of the ring carbons for compounds 1e-h and their σ-adducts in liquid ammonia at -55°C*

Compound	solvent	C-2	C-3	C-4	C-5	C-6
1e	D_2O	142.1	134.4	144.2	128.9	144.7
2e	NH_3	135.9	99.8	119.1	110.3	57.8
	Δδ	6.2	34.6	25.1	18.6	86.9
3e	NH_3	131.3	103.4	40.9	106.4	122.5
	Δδ	10.8	31.0	103.3	22.5	22.2
1f	D_2O	145.1	134.7	145.6	129.1	147.3
2f	NH_3	132.4	105.1	118.1	113.8	64.9
	Δδ	12.7	29.6	27.5	15.3	82.4
4f	NH_3	60.5	118.3	123.7	97.7	129.8
	Δδ	84.6	16.4	21.9	31.4	17.5
1g	D_2O	144.6	134.5	145.0	129.3	147.0
2g	NH_3	133.7	103.6	118.3	112.8	62.7
	Δδ	10.9	30.9	26.7	16.5	84.3
4g	NH_3	60.9	116.2	123.9	96.3	130.2
	Δδ	83.7	18.3	21.1	33.0	16.8
1h	D_2O	146.3	135.5	146.2	130.2	148.8
2h	NH_3	138.8	99.0	119.7	111.8	64.7
	Δδ	7.5	36.5	26.5	18.4	84.1
3h	NH_3	135.7	105.0	40.4	105.9	125.8
	Δδ	10.6	30.5	105.8	24.3	23.0
4h	NH_3	63.5	114.9	125.9	91.6	134.0
	Δδ	82.8	20.6	20.3	38.6	14.8

Their chemical shifts are assigned by comparison with those of the 1-*t*-butyl derivative and by using the chemical shifts of related 1,4-dihydro compounds[11].

Variation of the temperature from -70 to -20°C shows that the ratios C-2 adduct/C-6 adduct obtained from compounds 1f and 1g alter. In Table 4.6 this is illustrated for the σ-adducts obtained from 1f. The amount of C-2 adduct decreases in favour of the C-6 adduct at higher temperature. It is interesting to note that the ratio of the three σ-adducts obtained from 1h remains unaffected by temperature variation over this range. At a temperature above 0°C a fast reaction to 3-pyridinecarboxamide occurs with all three aryl compounds.

Table 4.6 Isomer distribution of σ-adducts from 3-aminocarbonyl-1-(pentadeuteriophenyl)pyridinium chloride 1f at various temperatures

T (°C)	C-6 adduct 2f (%)	C-4 adduct 4f (%)
-70	60	40
-45	65	35
-20	80	20

Comparing these results of covalent amination of 1f-h with those obtained for oxidation by aldehyde oxidase[2], it is evident that the similarity between these two reactions is very small (Table 4.3) and certainly less convincing as an "in vitro-model" than the corresponding reactions of the 1-alkyl derivatives (1a-e). This leads to the conclusion that in the 1-aryl compounds besides the steric influence of the aryl group, an electronic effect is operative which strongly influences the site of amination. The combined effect of steric and electronic effects makes comparison between σ-adduct formation and oxidation of limited value, since they probably operate in a different manner in both reactions.

4.3 EXPERIMENTAL SECTION

Melting points are uncorrected. ^{1}H NMR spectra were recorded on a Varian EM-390 spectrometer equipped with a Varian EM-3940 variable temperature controller using DSS as internal standard (δ = 0 ppm). Spectra in liquid ammonia were measured in sealed thickwalled NMR tubes. The proton chemical shifts in liquid ammonia were measured against the solvent signal (δ = 0.95 ppm). Isomer ratios

were determined by integration of appropriate signals. ^{13}C NMR spectra were recorded on a Bruker CXP 300 spectrometer equipped with a B-VT 1000 variable temperature controller. In D_2O dioxane (δ = 67.3 ppm) was employed as internal standard and in liquid ammonia a 3 mm capillary with acetone-d_6 was inserted, which was used both for the lock signal and as internal standard (δ = 29.8 ppm). Typical spectral parameters were: spectral width 15.000 Hz (1.85 Hz/point), acquisition time 0.27 s, pulse delay 1 s (C-H decoupled spectra) or 2 s (C-H coupled spectra) and pulse width 12 μs or 18 μs, respectively. Selective decoupling with 1e and 1g was carried out to check ^{13}C signal assignments. All NMR data were converted to the DSS scale by addition of the indicated values. The excess of ^{15}N in the compound investigated, was calculated from the (M+1)/M ratio, as determined on an AEI MS-902 spectrometer equipped with a VG ZAB console. Column chromatography was carried out over Merck silica gel 60 (70-230 mesh ASTM).

Preparation of starting materials

1-Alkyl-3-aminocarbonylpyridinium chlorides (1a-e)[1], *3-aminocarbonyl-1-(p-methoxyphenyl)pyridinium chloride (1g)*[2] *and 3-aminocarbonyl-1-(2,4,6-trimethylphenyl)pyridinium chloride (1h)*[2] were synthesized as described before. 4-Deuterated compounds of 1a, 1b, 1e and 1g were prepared according to the procedure of Caughey and Schellenberg[17]. NMR analysis indicated 85% deuteration of compound 1a, 58% of compound 1g (both after one oxidation-reduction cycle) and >95% of compounds 1b and 1e (three successive oxidation-reduction cycles).

3,14-Dioxa-18-methyl-18-azonia-bicyclo[14.3.1] eicosa-1(20), 16,18-triene-2,15-dione perchlorate (8) was a gift from Prof.Dr. R.M. Kellogg. ^{15}N-labelled ammonia was prepared by reacting ^{15}N-labelled ammonium nitrate (from VEB Berlin-Chemie) with potassium hydroxide.

3-Aminocarbonyl-1-(pentadeuteriophenyl)pyridinium chloride (1f)

This compound was obtained from the reaction of 3-aminocarbonyl-1-(2,4-dinitrophenyl)pyridinium chloride[18] with aniline-d_5 (> 99% deuteration; from Merck), according to the method described before[2]. Yield 81%, m.p. 253-254°C.
Anal.Calcd. for $C_{12}H_6D_5ClN_2O$: C, 60.12; H (+D), 6.72. Found: C, 59.89; H(+D), 6.67.

3-Aminocarbonyl-4-deuterio-1-(pentadeuteriophenyl)pyridinium chloride

Two methods have been employed to prepare this compound:
1. Direct introduction of deuterium in 1f[17], which gave a yield after one oxidation reduction cycle of 1%. ^{1}H NMR spectroscopy showed 70% deuteration.
2. An alternative procedure which involved first the introduction of deuterium[17] and secondly the introduction of the correct substituent on the ring nitrogen, using the ANRORC-mechanism[18].
3-Aminocarbonyl-4-deuterio-1-methylpyridinium chloride (4.4 g, 25 mmol) was dissolved in 50 ml of liquid ammonia and reacted in sealed Carius tubes at room temperature. After 4 days the tubes were opened, the ammonia evaporated and the residue dissolved in absolute ethanol. This mixture was refluxed for 15 min and subsequently the solvent was distilled off. The residue was purified by column chromatography, eluting with ethyl acetate/methanol (9:1). The yield of demethylated product[5a,b], 4-deuterio-3-pyridinecarboxamide (structure confirmed by ^{1}H NMR spectroscopy) was 58%. Reaction of this product to the desired compound was performed analogously to known synthetic procedures[2,18].
Starting with undeuterated 1a, the overall yield of the complete method was 26%. The 4-deuterium content of the end-product was 73%, as established by ^{1}H NMR analysis.

3,5-Dicarbethoxy-1-ethylpyridinium iodide (5)

Diethyl-3,5-pyridinedicarboxylate[19] (0.5 g, 2.2 mmol) was refluxed for 12 h with an excess of ethyl iodide in 10 ml of acetophenone. After evaporation of the solvent and recrystallization of the residue from acetone/ether 0.76 g of 5 was obtained (yield 90%), m.p. 167-168^{0}C.
Anal.Calcd. for $C_{13}H_{18}INO_4$: C, 41.17; H, 4.78. Found: C, 41.22; H, 5.10.

^{15}N-labelling of 3-pyridinecarboxamide

3-Aminocarbonyl-1-ethylpyridinium chloride (43 mg, 0.23 mmol) was reacted with 3 ml of liquid ammonia (8.1% of ^{15}N) according to the procedure described above. Yield 93%. The ^{15}N content of isolated 3-pyridinecarboxamide at N-1 was 8.0%.

Acknowledgements

We wish to thank Prof.Dr. R.M. Kellogg (State University of Groningen) for the generous gift of crown ester, Prof.Dr.F.Müller for his critical reading of the manuscript, Drs.C.A.Landheer and Mr.C.J.Teunis for carrying out mass spectroscopy, Mr.H.Jongejan for the microanalyses and Dr.H.A.J.Holterman for measuring some ^{13}C NMR spectra.

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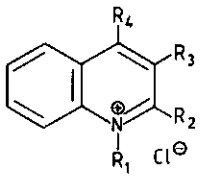
5 THE OXIDATION OF 1-ALKYL(ARYL)QUINOLINIUM CHLORIDES BY ALDEHYDE OXIDASE

5.1 INTRODUCTION

The oxidation of azaheterocyclic compounds with the molybdenum iron-sulphur containing flavoproteins xanthine oxidase[1] from bovine milk and aldehyde oxidase[1] from rabbit liver for synthetic purposes is a current subject of investigation in our laboratories[2,3,4]. Rabbit liver aldehyde oxidase has been successfully used to oxidize a number of 1-alkyl(aryl)-3-aminocarbonylpyridinium chlorides[2,3] under mild conditions. We established that the site of oxidation in these compounds is greatly affected by the substituent on the ring nitrogen atom. We also found evidence, by studying the influence of steric and electronic effects of the substituent on the maximum rate of oxidation, that in the catalytic mechanism the nucleophilic attack is the rate-limiting step[3].
As an extension of this work we report in this paper on the oxidation of 1-alkyl(aryl)quinolinium compounds (Scheme 5.1) by rabbit liver aldehyde oxidase. These compounds can be regarded as extended pyridinium analogues and therefore very probably will be oxidized in the same manner with regard to the influence of the N-1 substituent.



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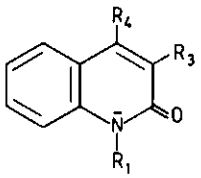

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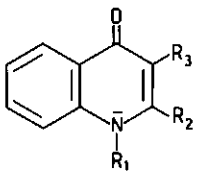


3

a : $R_1 = CH_3$, $R_2 = R_3 = R_4 = H$
b : $R_1 = CH_2C_6H_5$, $R_2 = R_3 = R_4 = H$
c : $R_1 = CH_2$[2,6-di-Cl-C_6H_3], $R_2 = R_3 = R_4 = H$
d : $R_1 = CH_3$, $R_3 = CONH_2$, $R_2 = R_4 = H$
e : $R_1 = CH_2C_6H_5$, $R_3 = CONH_2$, $R_2 = R_4 = H$
f : $R_1 = R_2 = CH_3$, $R_3 = R_4 = H$
g : $R_1 = R_4 = CH_3$, $R_2 = R_3 = H$

Scheme 5.1

Furthermore we present the results of our study on covalent amination of compounds 1, assumed to serve as a model reaction for the rate-limiting covalent nucleophilic addition occurring at the active site of the enzyme[5].

5.2 RESULTS AND DISCUSSION

5.2.1 Enzymic oxidation of 1-alkyl(aryl)quinolinium chlorides

When substrates 1a-c are incubated with the enzyme and the reaction mixture is analyzed by HPLC, it was observed that the N-1 substituent has a pronounced effect on the site of oxidation. 1-Methylquinolinium chloride (1a) is oxidized mainly at C-2, yielding 1-methyl-2(1H)-quinolinone (2a); only a small amount of 1-methyl-4(1H)-quinolinone (3a) is formed. Oxidation of 1-benzylquinolinium chloride (1b) gives mainly 4-oxo compound 3b and 2-oxo compound 2b only in a small amount. Compound 1-(2,6-dichlorobenzyl)quinolinium chloride (1c) is exclusively oxidized at C-4. The quinolinones 2a and 3a-c are identified by comparison with independently prepared samples; 2b has been identified after isolation by UV spectroscopy, exact mass determination and melting point. The maximum rates of oxidation at the different sites of the compounds 1a-c were measured at pH=9.0 and the results are collected in Table 5.1.

Table 5.1 Maximum rates for the oxidation of 1-alkyl(aryl)quinolinium chlorides 1a-g by free aldehyde oxidase at pH=9.0[a]

Substrate	$V_{2\text{-oxo}}$[b]	$V_{4\text{-oxo}}$[b]
1a	0.66 ± 0.03[c]	[d]
1b	0.043 ± 0.005	0.34 ± 0.03
1c		0.63 ± 0.04
1d		[d]
1e		0.015 ± 0.002
1f		0.014 ± 0.002
1g	0.180 ± 0.011	

a. The maximum oxidation rate for the reference substrate 3-aminocarbonyl-1-methylpyridinium chloride[2] with this aldehyde oxidase preparation was 0.42 ± 0.02 μmol/min mg.

b. In μmol/min mg.

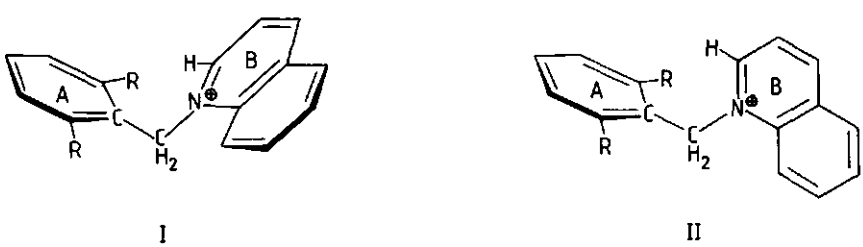
c. The very small contribution of the rate of oxidation at C-4 was neglected.

d. No accurate rate data could be determined for oxidation at this site.

It shows that in 1a the maximum rate of oxidation at C-2 is about 15 times higher than that at C-2 in 1b. In contrast, the rate of oxidation at C-4 in 1a is very low (no accurate data could be obtained) and the rate of the exclusive oxidation at C-4 in 1c is about two times higher than that at C-4 in 1b. With 1b the maximum rate of oxidation at C-2 is 8 times lower than at C-4. These changes in site of oxidation combined with the corresponding rates of oxidation can most likely be ascribed to a change of orientation of the substrates in the active site of the enzyme when the size of the substituent has increased. This has been observed before in the aldehyde oxidase-mediated oxidation of 3-aminocarbonyl-1-(2,4,6-trimethylphenyl)pyridinium chloride[3].

To obtain some information about the altered steric conformation of compound 1c in comparison to 1b, we measured the ^{1}H NMR spectra of compounds 1b and 1c and calculated the $\Delta\delta(H_2 - H_4)$ values. These $\Delta\delta$ values reflect the steric influence of the N-1 substituent since the electronic effects of these substituents on the chemical shifts of H-2 and H-4 are almost equal.

The $\Delta\delta(H_2 - H_4)$ value found was 0.29 ppm for 1b and -0.42 ppm for 1c, indicating a strong shielding effect of the 2,6-dichlorobenzyl group at C-2[6]. This results in a net upfield shift of about 0.7 ppm. Two extreme conformations, in which both ring planes A and B face each other (I) or are at right angles (II), are shown in Figure 5.1[7]. These conformations are transformed into one another by a 90° rotation of ring B about the C-N bond. As a consequence of steric hindrance it is to be expected that, especially for R = Cl, conformation II will be more favourable than I, leading to the shielding effect of the H-2 of the quinolinium ring by the 2,6-dichlorobenzyl group.



I II

Figure 5.1 Two conformations of compounds 1b and 1c resulting from rotation about the C-N bond.

Similar results were acquired before for 3-aminocarbonyl-1-(2,4,6-trimethylbenzyl)pyridinium chloride[3]. We suggest that the orientation of the 2,6-dichlorobenzyl group as indicated in Figure 5.1 by conformation II will be at least partly retained in the catalytic site of the enzyme, which for substrate 1c results in an exclusive oxidation at C-4. The results obtained with 1-methyl- and 1-benzylquinolinium chloride clearly show that the presence of an electron-withdrawing substituent such as an aminocarbonyl group in the pyridine moiety is not a requirement for oxidation. In contrast the analogues 1-methyl- and 1-benzylpyridinium chloride do need the presence of such a group at C-3[8]. The enhanced accessibility of the quinolinium ring for oxidation in comparison to the pyridinium ring is due to the fact that formation of the intermediate adduct, as assumed in the enzyme model[3] requires less energy in the case of quinolinium salts[9].

It was of interest to observe that the presence of an aminocarbonyl group at C-3 in substrates 1d and 1e directs the oxidation completely to C-4 irrespective whether the N-1 substituent is a methyl- or a benzyl group. In spite of the electron-withdrawing effect of the substituent at C-3, the maximum oxidation rates are very low for both compounds (Table 5.1). In fact no accurate maximum rate determination is possible for substrate 1d. Apparently the substrate molecule is oriented in the active site of the enzyme in such a way that the molecule is no longer accessible for the nucleophilic species responsible for the initial attack at C-2. It has been established that interaction of the C=O moiety of the aminocarbonyl group with a (proton-donating) species in the active site is an important factor in determining the orientation of the substrate molecules in the closely related enzyme xanthine oxidase from bovine milk[10].

Oxidation of the 1,2-dimethylquinolinium salt 1f gave exclusive formation of 1,2-dimethyl-4(1H)-quinolinone (3f). If however the 1,4-dimethylquinolinium compound 1g is oxidized, the oxidation only takes place at C-2. The maximum oxidation rates with both quinolinium chlorides 1f and 1g are lower than those of the parent compound 1a (Table 5.1).

The low rate for the 1,2-dimethylquinolinium chloride 1f is of course due to the fact that the favoured site C-2 is now blocked by the presence of a methyl group. Moreover, the electron-donating character of the methyl group at C-2 diminishes the rate of oxidation at C-4, which is very low in compound 1a, as we have already mentioned. This effect is also operative in the oxidation of 1,4-dimethylquinolinium chloride which shows a maximum rate of about 3.7 times

lower than the rate for 1-methylquinolinium chloride. In the nucleophilic substitution reaction of 2-chloroquinoline with methoxide ions a decrease in reaction rate of a factor 2.5 was established by the introduction of a methyl group at C-4[11]. It is obvious from this latter reaction that the electron-donating effect of the methyl substituent at C-4 lowers the enzymic oxidation rate considerably and suggests that the nucleophilic attack is the rate-limiting step in the oxidation of these substrates as well. The maximum oxidation rate for substrate 1a has been determined over the pH range 6.4-9.7. The rate gradually increases from pH 6.4 to 7.7 and is about constant over the pH range 7.7-9.7. This pH profile is quite similar to that found for 3-aminocarbonyl-1-phenylpyridinium chloride[3]. With immobilized aldehyde oxidase[2] oxidation of the quinolinium salts 1a-c to the respective quinolinones was performed on a small preparative scale[12]. Total product yields varying from 34 to 48% have been obtained (Table 5.4); these yields are rather low compared to those acquired in the conversions of 3-aminocarbonyl-1-arylpyridinium chlorides[3].

5.2.2 Covalent amination

In a previous paper[5] we described the addition of liquid ammonia to 1-alkyl(aryl) 3-aminocarbonylpyridinium chlorides as a "model" for the formation of the initial

Table 5.2 ^1H NMR data of the protons of the pyridine ring moiety for σ-adducts of compounds 1a, 1d, 1f and 1g in liquid ammonia at -45°C[a]

Compound	H-2	Δδ[b]	H-3	Δδ[b]	H-4	Δδ[b]	$N\text{-}CH_3$	Δδ[b]
4a	4.65	4.69	5.82	2.26	6.54	2.62	2.89	1.85
4f[c]			5.62	2.36	6.43	2.49	2.89	1.63
4g[d]	4.55	4.59	5.65	2.33			2.87	1.77
4d	5.05	4.63			f	-	2.98	1.76
5[e]	f	-			4.83	4.69	3.31	1.43

a. Adduct 4a: $J_{2,3}$ = 5.4 Hz, $J_{3,4}$ = 9.6 Hz; 4f: $J_{3,4}$ = 9.6 Hz; 4g: $J_{2,3}$ = 5.4 Hz.

b. Upfield shifts relative to the corresponding compounds 1 in D_2O.

c. C-2(CH_3): 1.69 ppm (Δδ = 1.45).

d. C-4(CH_3): 1.96 ppm (Δδ = 1.05).

e. After incubation at room temperature.

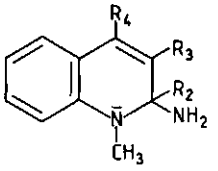
f. Not interpretable due to overlap by the proton signals of the benzo ring.

intermediate during the catalytic reaction of aldehyde oxidase[3]. The "covalent amination-model" was found to match the oxidation pattern of the enzyme quite well in the case of the 1-alkyl-3-aminocarbonylpyridinium chlorides and to a lesser degree for the 1-aryl derivatives. Therefore we investigated the utility of this model-system for some of the quinolinium substrates described in this paper. In Table 5.2 the ^{1}H NMR data of the addition products formed between the substrates 1a, 1d, 1f, 1g and liquid ammonia are collected. The assignment of signals for the addition products 4a, 4f and 4g is based on the upfield shifts for the protons of the pyridine ring moiety and the blocking of potential addition sites by methyl substituents. The structure of adducts 4d and 5 was established by measuring the σ-adducts formed between 3-aminocarbonyl-*4-deuterio*-1-methylquinolinium chloride and liquid ammonia. In addition ^{13}C NMR data were collected for these latter adducts and the parent compound 1d (Table 5.3), which were compared with corresponding data of 1-alkyl(aryl)-3-aminocarbonylpyridinium chlorides and their σ-adducts[5].

In Table 5.2 it is clearly shown that the covalent amination of compounds 1a, 1f and 1g takes place only at C-2[13], resulting in the corresponding adducts 4 (Scheme 5.2). It is interesting however that, despite the presence of a methyl group at C-2, the 1,2-dimethylquinolinium salt 1f also exhibits addition at C-2[14]. This is reflected by the larger upfield shift of the proton signal of the 2-methyl group in adduct 4f in comparison to the corresponding shift of the 4-methyl group in adduct 4g (Table 5.2). At room temperature adducts 4a, 4f and 4g are unstable; most likely they undergo ring-opening reactions[5].

The results indicate that the presence of an electron-withdrawing substituent at C-3 in quinolinium salts is not required for amination. These compounds differ from 1-methyl- and 1-benzylpyridinium chloride with respect to the amination reaction[15]. Compound 1c does not give any adduct at -45°C. Obviously the steric orientation of the N-1 substituent prevents nucleophilic attack on C-2 by ammonia. The important influence of steric effects on covalent amination in liquid ammonia has been established before[5].

Covalent amination of compound 1d at -45°C gives the C-2 adduct 4d. At higher temperatures (> -40°C) a second adduct is formed, viz. 5 and at room temperature both adducts are present in a ratio of 35:65, respectively. The assignment of adduct structures is also verified by ^{13}C NMR (Table 5.3), which shows good agreement with former data obtained for 1-alkyl(aryl)-3-aminocarbonylpyridinium chlorides in liquid ammonia[5]. Apparently 4d is a kinetically favoured adduct which at higher temperature partly converts into its isomer 5.



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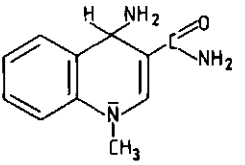

5

a : $R_2 = R_3 = R_4 = H$
d : $R_3 = CONH_2$, $R_2 = R_4 = H$
f : $R_2 = CH_3$, $R_3 = R_4 = H$
g : $R_4 = CH_3$, $R_2 = R_3 = H$

Scheme 5.2

Table 5.3 *^{13}C NMR data of some carbon atoms in compound 1d and its σ-adducts in liquid ammonia*

Compound	Solvent	C-2	C-4	CH_3
1d	dmso-d_6	149.8	145.3	45.9
4d[a]	NH_3	66.0	127.7	35.2
	Δδ	83.8	17.6	10.7
5[b]	NH_3	138.9	46.3	38.7
	Δδ	10.9	99.0	7.2

a. At -50°C.

b. After incubation at room temperature.

The formation of two adducts has also been found in the reaction of 1-benzyl-3-cyanoquinolinium bromide with hydroxide ions[16]. Adducts 4d and 5 are stable for at least 24 hours in liquid ammonia at room temperature. Comparison of the sites of oxidation in compounds 1a, 1d, 1f-g with those of covalent amination clearly shows a great diversity. The "covalent amination model" does not predict oxidation at C-4 with compounds 1a and 1f since the formation of σ-adducts occurs predominantly at C-2 irrespective of the methyl substituent present at the carbon atom. In the presence of a 3-aminocarbonyl group addition takes place at C-2 and C-4, whereas oxidation only occurs at C-4. Therefore we have to conclude that covalent amination in liquid ammonia is not a good model

for description of the covalent addition step in the oxidation of 1-alkyl(aryl) quinolinium salts by aldehyde oxidase.

5.3 EXPERIMENTAL SECTION

Melting points are uncorrected. Mass spectra were determined on an AEI MS-902 mass spectrometer equipped with a VG ZAB console. The ^{1}H NMR spectra were recorded on a Varian EM-390 spectrometer equipped with a Varian EM-3940 variable temperature controller with DSS or TMS as internal standard (δ = 0 ppm). Spectra in liquid ammonia were measured in sealed thick-walled NMR tubes. The proton chemical shifts in liquid ammonia were measured against the solvent signal (δ = 0.95 ppm). Isomer ratios were determined by integration of appropriate signals. ^{13}C NMR spectra were recorded on a Bruker CXP 300 spectrometer equipped with a B-VT 1000 variable temperature controller. In liquid ammonia a 3 mm capillary with acetone-d_6 was inserted, which was used both for the lock signal and as internal standard (δ = 29.8 ppm). Typical spectral parameters were: spectral width 15.000 Hz (1.85 Hz/point), acquisition time 0.27 s, pulse delay 1 s (C-H decoupled spectra) or 2 s (C-H coupled spectra) and pulse width 15 µs or 18 µs, respectively. All NMR data were converted to the DSS/TMS scale.
HPLC analysis was performed with a Varian 5000 instrument equipped with a Micro Pak MCH-10 column (30 x 0.4 cm), a Schoeffel GM-770 monochromator and an SF-770 spectroflow monitor. Operating conditions were Δp=19.4 MPa (2814 psi), eluent water-methanol 50:50 (v/v) and flow rate 2.0 ml/min.
UV spectra and kinetic assays were determined on an Aminco DW-2a UV/VIS spectrophotometer. Column chromatography was carried out over Merck silica gel 60 (70-230 mesh ASTM). Partially purified aldehyde oxidase (E.C.1.2.3.1) was prepared from frozen rabbit liver as described previously[2]. This type of preparation was used for kinetic assays. For synthetic purposes a less-purified preparation, acquired by ommission of the hydroxylapatite step in the purification procedure, was employed. Rabbit liver xanthine oxidase (E.C.1.2.3.2) was isolated as described previously[3].

Preparation of starting materials and reference compounds

The quinolinium chlorides 1 were prepared from the corresponding bromides or iodides by passage over a Dowex 1-X2 column.
1-R-quinolinium (R = methyl(1a)[17]*, benzyl(1b)*[18]*, 2,6-dichlorobenzyl(1c))*[19]*, 1,2-dimethylquinolinium(1f)*[20]*, 1,4-dimethylquinolinium(1g)*[21]*, 3-aminocarbonyl-1-R-quinolinium (R = methyl(1d), benzyl(1e))*[22] *bromide or iodide, 3-aminocarbonyl-1-methylpyridinium chloride*[2]*, 3-aminocarbonyl-4-deuterio-1-methyl-*

quinolinium chloride (74% deuteration according to NMR analysis)[23], *1-methyl-2-(1H)-quinolinone(2a)*[24], *1,4-dimethyl-2(1H)-quinolinone (2g)*[25], *1-methyl-4(1H)-quinolinone (3a)*[26] *and 1,2-dimethyl-4(1H)-quinolinone (3f)*[27] were synthesized according to known synthetic procedures. 4(1H)-Quinolinone trihydrate was purchased from Aldrich.

1-R-4(1H)-quinolinone (R = benzyl(3b), 2,6-dichlorobenzyl(3c))

4(1H)-Quinolinone trihydrate (0.7 g, 3.5 mmoles) was dissolved in 20 ml of methanol containing 0.5 g (12.5 mmoles) sodium hydroxide. To this solution 21.5 mmoles of the appropriate arylbromide (benzylbromide, α-bromo-2,6-dichlorotoluene) were added and the mixture was refluxed for one hour. The precipitate formed was filtered off and the filtrate evaporated to dryness. The residue was crystallized from absolute ethanol-ether (R = benzyl) or purified by column chromatography (R = 2,6-dichlorobenzyl) with dichloromethane-ethyl acetate (1:1) as eluent followed by crystallization from ethanol. R = benzyl: yield 48%; m.p. 123-124°C (lit.[28] 124-125°C). R=2,6-dichlorobenzyl: yield 60%; m.p. 198.5-199.5°C.
Anal. Calcd. for $C_{16}H_{11}Cl_2NO$: C, 63.18; H, 3.64. Found: C, 62.88; H, 3.65.

1-R-1,4-dihydro-4-oxo-3-quinolinecarboxamide (R = methyl(3d), benzyl(3e))

1-R-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid[26] was converted to the corresponding aminocarbonyl compound according to the method of Tanaka and Price[29]. The acid (9.9 mmoles) was dissolved in a mixture of 10 ml of chloroform and 20 ml of tetrahydrofuran containing 2 g (19.8 mmoles) of triethylamine. To this solution 2 g (18.4 mmoles) of ethyl chloroformate was added dropwise with stirring at -5°C. After one hour 30 ml of 33% ammonia was added, the precipitate filtered off (yield 80%, R = methyl or benzyl) and recrystallized twice from ethanol. R = methyl: m.p. 286-288°C.
Anal. Calcd. for $C_{11}H_{10}N_2O_2$: C, 65.33; H, 4.98. Found: C, 65.22; H, 4.82.
R = benzyl: m.p. 232-233°C.
Anal. Calcd. for $C_{17}H_{14}N_2O_2$: C, 73.36; H, 5.07. Found: C, 73.52; H, 4.79.

HPLC analysis

The HPLC analysis of the reaction mixtures was performed as described previously[2]. Registration of the oxidation products occurred by UV detection at 254 nm (log ε for 2a: 3.60; 3a: 3.57; 2b: 3.51; 3b: 3.61; 3c: 3.63; 3d: 4.33; 3e:4.31; 3f: 3.37; 2g: 3.57).

Synthesis of the quinolinones 2a, 2b, 3a-c with immobilized aldehyde oxidase

Rabbit liver aldehyde oxidase was immobilized by adsorption onto DEAE Sepharose CL 6B as described before[2], applying 45 mg of aldehyde oxidase-containing protein per 3.5 ml packed Sepharose gel during immobilization. The immobilized enzyme preparation was packed in a column and washed with 10 mM phosphate buffer, pH=7.8 (0.1 mM EDTA) at 4°C. For each conversion 13 units of aldehyde oxidase were used (for definition see *Kinetic assays*). The DEAE Sepharose was regenerated after depletion of the aldehyde oxidase activity as described elsewhere[30]. 200 to 350 ml of a 0.5 mM substrate solution in the same buffer (32 mg of substrate applied) was slowly (0.25 ml/min) recirculated through the column at 4°C with a pump and the formation of product(s) was registrated at 254 nm (HPLC). After depletion of the aldehyde oxidase activity, the collected effluent was brought to about pH=6.5, evaporated to dryness and the residue purified by column chromatography (eluents dichloromethane and ethyl acetate). UV and mass spectra of 2a and 3a-c were identical to those of authentic materials. For compound 2b no authentic sample was available; after isolation the melting point was identical with that reported. The yields and exact mass measurement data of the crude products are summarized in Table 5.4.

Table 5.4 Product yields and exact mass data of some 1-alkyl(aryl)-quinolinones obtained by oxidation using immobilized aldehyde oxidase

Product	Yield (%)	Formula	Exact mass	
			Experimental	Theoretical
2a	40	$C_{10}H_9NO$	159.0684	159.0684
3a	<3	$C_{10}H_9NO$	159.0683	159.0684
2b[a]	11	$C_{16}H_{13}NO$	235.1006	235.0997
3b	37	$C_{16}H_{13}NO$	235.1009	235.0997
3c	34	$C_{16}H_{11}Cl_2NO$	303.0222	303.0219

a. After recrystallization from ethanol m.p. 50-51°C (lit.[31] 50-51°C).

Kinetic assays

The assay for aldehyde oxidase was carried out as described previously[2]. Each assay was at least performed in duplicate. As buffer sodium borate, pH=9.0 with an ionic strength I = 0.05, including 0.1 mM EDTA, was used. For assays in the pH range 6.4-9.7 buffers of the same ionic strength were employed[32]. In the case where the formation of two products from one single substrate was monitored, the initial reaction rate was measured at two suitable wavelengths. The appropriate wavelengths (λ in nm) and corresponding molar differential absorption coefficients (log $\Delta\varepsilon$) are: 1a-2a: 273 (3.72); 1b-2b: 273 (3.69), 323 (3.19); 1b-3b: 273 (-0.60), 323 (3.96); 1c-3c: 323 (3.89); 1e-3e: 305 (3.87); 1f-3f: 323 (3.41); 1g-2g: 271 (3.76). These parameters are constant over the pH range studied, *i.e.* 6.4-9.7. For aldehyde oxidase one unit of enzyme activity is defined as the amount of enzyme which oxidized 1 µmol of 3-aminocarbonyl-1-methylpyridinium chloride per min at 25°C. The assay conditions were: 5 mM substrate in 50 mM potassium phosphate buffer, pH=7.8 (0.1 mM EDTA) with the reaction being monitored at 292 nm (log $\Delta\varepsilon$= 3.64). Kinetic data were calculated from Lineweaver - Burk plots[33].

Acknowledgements

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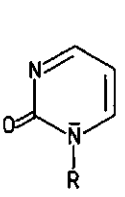
6 THE OXIDATION OF N-METHYL- AND N-BENZYL-PYRIMIDIN-2- AND -4-ONES BY ALDEHYDE OXIDASE

6.1 INTRODUCTION

The application of immobilized enzymes in organic synthesis is a subject of current interest in our laboratories. The molybdenum iron-sulfur containing flavoproteins xanthine oxidase/dehydrogenase[1] isolated from bovine milk, chicken liver or Arthrobacter cells and aldehyde oxidase[1] obtained from rabbit liver were employed as immobilized biocatalysts in the oxidation of several six-membered azaheterocyclic compounds[2-5]. These enzymes are of particular interest since they possess broad substrate specificities[1]. The oxidation of heteroaromatic cations such as 1-alkyl(aryl)-3-aminocarbonylpyridinium chlorides[3,4] and 1-alkyl(aryl)-quinolinium chlorides[5] was investigated using aldehyde oxidase in particular.



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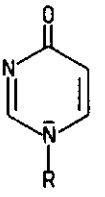

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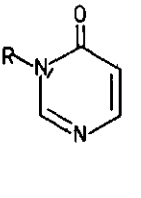

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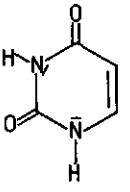

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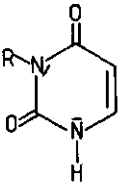


5

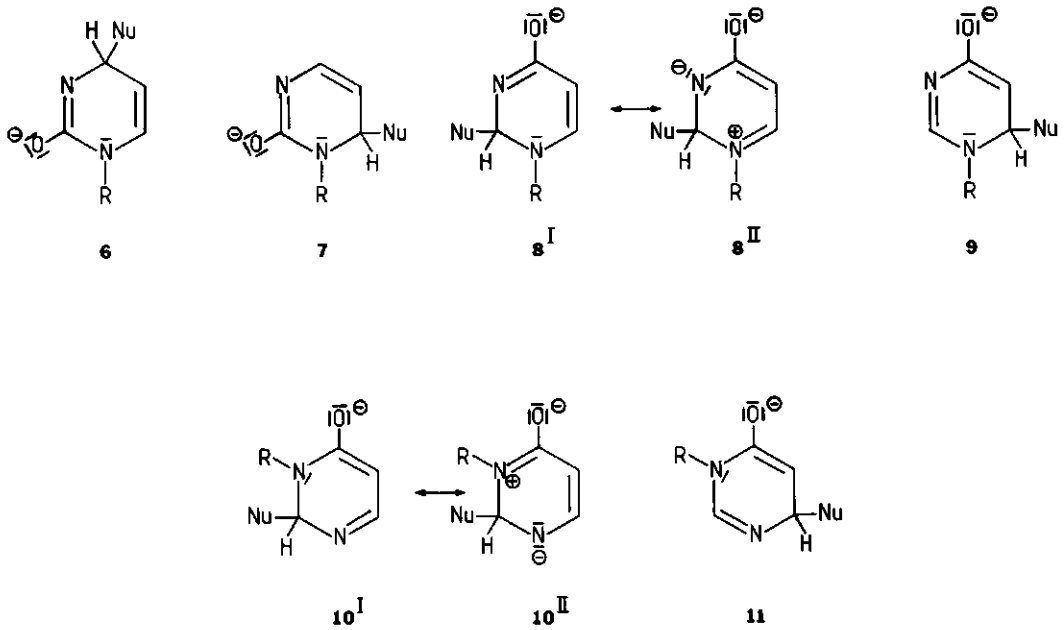
R = a : H , b : CH_3 , c : $CH_2C_6H_5$

Scheme 6.1

A marked influence of the size and the electronic effect of the substituent at the ring nitrogen atom on the site(s) of oxidation in these compounds was established.
Up to now the reactivity of aldehyde oxidase towards uncharged substrates has hardly been investigated with regard to the influence of N-substituents on the site of oxidation[6]. We therefore extended our research to the aldehyde oxidase-mediated oxidation of N-methyl- and N-benzylpyrimidin-2- and -4-ones (Scheme 6.1).

6.2 RESULTS AND DISCUSSION

It is known that the compounds 2(1H)-pyrimidinone and 4(3H)-pyrimidinone are readily oxidized by aldehyde oxidase[6]. Analysis with HPLC showed that substrates 1a-c and 2a-c only give formation of the corresponding uracil derivatives 4a-c. Substrates 3b-c yield the N-3 substituted compounds 5b-c as sole products. In all these oxidation reactions no indication is found for the simultaneous formation of products isomeric with 4 or 5 and no barbituric acid or its derivatives are detected either. This is confirmed by incubation of compounds 4 and 5 with aldehyde oxidase; no conversion into barbituric acid or its derivatives is observed.



Scheme 6.2

The results indicate that in substrates 1b and 1c the position adjacent to the unsubstituted nitrogen atom (C-4) is preferred to the position adjacent to the substituted nitrogen atom (C-6) for oxidation. To understand these oxidation patterns we may consider the intermediates formed on addition of the active site nucleophilic species[4] to these substrates (Scheme 6.2). Nucleophilic attack on substrates 1a-c can take place either at C-4, yielding intermediates 6a-c or at C-6, forming adducts 7a-c. Intermediate 6 possesses a p-quinoid structure and intermediate 7 an o-quinoid structure.

Since in general the p-quinoid structure is more stabilized than the corresponding o-quinoid structure[7], it is comprehensible that 1a-c are oxidized at the C-4 position. For 4-pyrimidinone it is well documented that the o-quinoid structure is more stable than the p-quinoid structure[8]. It is expected that from the two possible adducts 10 and 11 obtained from 3, adduct 10 has the most preferred structure:

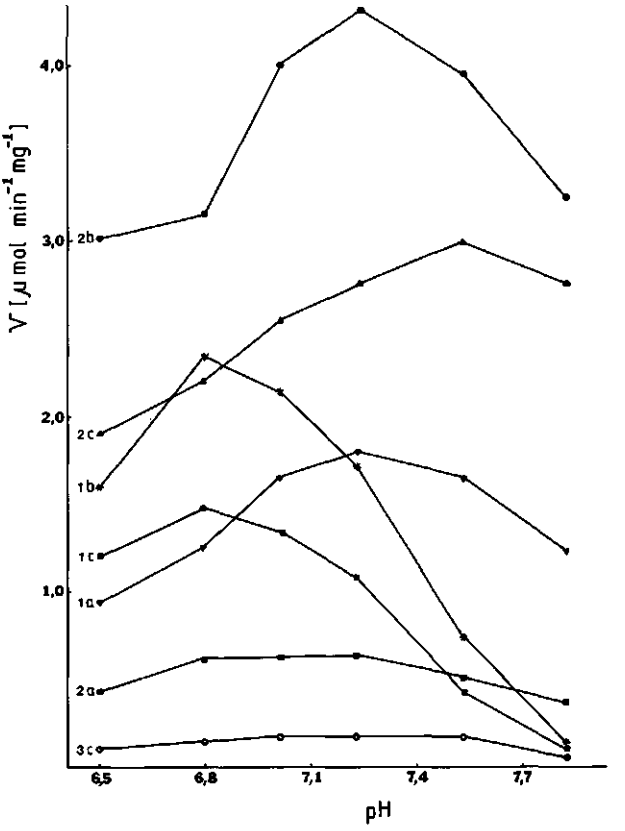


Figure 6.1 Maximum rates for the oxidation of substrates 1a-c, 2a-c and 3c by free aldehyde oxidase as a function of pH at 25 °C.

in adduct 10 there is more extended conjugation between both nitrogen atoms than in 11, thus favouring 10 to 11. The addition of the nucleophile to 2 predicts preferred formation of adduct 8 to 9, since 8 is more resonance-stabilized than 9.

These results are also in agreement with the observations that the presence of a nucleophilic substituent at C-4 deactivates a nucleophilic attack at C-6 in favour of C-2: for instance 2,6-dichloro-4-methoxypyrimidine only undergoes dechlorination at C-2 by methoxide ions[7].

The maximum oxidation rates for substrates 1a-c, 2a-c and 3c with free enzyme are determined as a function of the pH in the pH-region 6.5-7.8 (Figure 6.1). For compound 3b we found a rather low oxidation rate at pH=7.0 and therefore we did not determine the maximum rate for this substrate over the whole pH range. All substrates studied show a pH-optimum in the pH-region of 6.5-7.8. The pH-optima for substrates 1a-c shift from about pH=7.2 for compound 1a to pH=6.8 for 1b and 1c. For the 4-pyrimidinones substituted at N-1 the optimum shifts to slightly higher pH values, whereas substitution at N-3 does not affect the pH-optimum significantly. From these observations we conclude that the effect of N-substitution by a methyl or a benzyl group on the pH-optimum of the maximum rate is rather small.

For the pyrimidinones 1a and 2a the maximum rate of oxidation is found to decrease at higher pH, due to deprotonation (lit.[9] 1a: pK_a= 9.37 and 2a: pK_a= 8.60) leading to negatively charged species which are deactivated for nucleophilic attack[4].

It is interesting that on comparison of the results of these pH dependencies of the oxidation rates with those obtained for the cationic substrates studied before[4,5], a remarkable difference in the shape of the pH curves is observed. 3-Aminocarbonyl-1-phenylpyridinium chloride[4] and 1-methylquinolinium chloride[5] for instance, exhibit a nearly constant maximum rate above pH=7.5, whereas we here find bell-shaped pH curves for all substrates.

The magnitude of the maximum rates is strongly dependent on the nature and the position of the N-substituent. Upon methylation or benzylation at N-1 of 4(3H)-pyrimidinone the maximum rate increases drastically. This effect is comparable to that obtained upon introduction of a methyl group in hypoxanthine at the equivalent N-3 position[6]. Introduction of substituents at N-3 of 4(3H)-pyrimidinone results, irrespective of the size of the substituent, in a large decrease of the maximum rates (Table 6.1) over the whole pH-range. These results can be partly explained by comparing the initial and intermediate structures of

the substrate in the oxidation reactions. Compounds 2b-c both possess the p-quinoid structure, but intermediates 8b-c with their azadiene o-quinoid structure are strongly resonance-stabilized. This will facilitate the initial nucleophilic attack at C-2 in the oxidation of these substrates.

Table 6.1 Kinetic data for the oxidation of substrates 1a-c, 2a-c and 3b-c by free aldehyde oxidase at pH=7.0[a]

Substrate	K_M [b,d]	V [c,d]
1a	244	1.63
1b	272	2.13
1c	78	1.33
2a (=3a)	543	0.62
2b	185	3.98
2c	66	2.51
3b	156	0.052
3c	61	0.158

a. The maximum oxidation rate for the reference substrate 3-aminocarbonyl-1-methylpyridinium chloride[3] with this aldehyde oxidase preparation was 0.36 ± 0.02 µmol/min.mg.

b. In µmol/l.

c. In µmol/min.mg.

d. Accuracy for 2a, 3b-c was 4%, for 1a-b 7% and for 3c 14%.

The corresponding step in the oxidation of 2a, which is present in the o-quinoid form 3a[8] involves intermediate 10a and the adduct formation certainly requires a higher transition state energy than for 2b-c. For 3b-c the initial structures are o-quinoid and therefore no rate enhancement could be expected in comparison with the oxidation rate for 3a. On the contrary a substantial decrease in maximum rate has been observed for both compounds. It is apparent that other effects must play an important role and a likely possibility is that substrates 3b-c are oriented differently in the active site of the enzyme because of interaction of the hydrophobic group at N-3 with the proposed hydrophobic region in the enzyme[3], possibly making C-2 less accessible for nucleophilic attack. Support for this suggestion comes from the considerably lower Michaelis constant values (K_M) observed upon the introduction of large hydrophobic substituents

(Table 6.1).
For 2(1H)-pyrimidinone and its derivatives the situation is less clear. At low pH the reasoning used for 1-R-4(1H)-pyrimidinones also shows some validity here, but above pH=7.2 (R=methyl) and pH=6.9 (R=benzyl) the N-substituted 2(1H)-pyrimidinones are oxidized at lower rates than 2(1H)-pyrimidinone itself. Obviously other effects in the active centre of the enzyme play a predominant role in the oxidation of these compounds at higher pH.
Oxidation of substrates 1b-c, 2b-c and 3b-c on a small preparative scale is performed with immobilized aldehyde oxidase. The enzyme preparation used for this purpose contained a low indigenous xanthine oxidase activity as well. Therefore we isolated xanthine oxidase from rabbit liver and tested the activity of this enzyme on these substrates. We established by HPLC analysis only very small activities of xanthine oxidase for substrates 1b and 1c, which are oxidized into the uracil derivatives 4b and 4c, respectively. The other substrates (2b-c, 3b-c) are not converted by this enzyme. Consequently the yields in Table 6.2 for products 4b-c derived from substrates 1b-c may contain a very small contribution from the xanthine oxidase-mediated reaction.
The product yields found are comparable with those obtained in the oxidation of 1-alkyl(aryl)quinolinium chlorides[5], although the slowly converted substrates 3b-c give a significantly higher product yield. To obtain the products 4b-c by this method the oxidation of substrates 2b-c is slightly more profitable than starting from substrates 1b-c.

6.3 EXPERIMENTAL SECTION

Mass spectra were determined on an AEI MS 902 mass spectrometer equipped with a VG ZAB console. HPLC analysis was performed with a Varian 5000 instrument equipped with a Micro Pak MCH-10 column (30 x 0.4 cm), a Schoeffel GM-770 monochromator and an SF-770 spectroflow monitor. Operating conditions were Δp=14.8 MPa (2146 psi), eluent water-methanol 80:20 or 70:30 (v/v) and flow rate 1.6 ml/min. UV spectra and kinetic assays were determined on an Aminco DW-2a UV/VIS spectrophotometer. Column chromatography was carried out over Merck Silica gel 60 (70-230 ASTM). Partially purified aldehyde oxidase (E.C. 1.2.3.1) was prepared from frozen rabbit livers as described previously[3]. This type of preparation was used for kinetic assays. A less-purified preparation, acquired by ommission of the hydroxylapatite step in the purification procedure, was employed for synthetic purposes. Rabbit liver xanthine oxidase (E.C. 1.2.3.2) was isolated as described before[4].

Starting materials and reference compounds

The following compounds were synthesized according to procedures described in the literature: *4(3H)-pyrimidinone (2a)*[10], *1-methyl-4(1H)-pyrimidinone (2b)*[10], *1-benzyl-4(1H)-pyrimidinone (2c)*[10], *3-methyl-4(3H)-pyrimidinone (3b)*[10], *3-benzyl-4(3H)-pyrimidinone (3c)*[10], *1-methyl-2(1H)-pyrimidinone (1b)*[11], *1-benzyl-2(1H)-pyrimidinone (1c)*[12], *1-methyluracil (4b)*[9], *3-methyluracil (5b)*[9], *1-benzyluracil (4c)*[13], *3-benzyluracil (5c)*[14], *3-aminocarbonyl-1-methylpyridinium chloride*[3]. 2(1H)-Pyrimidinone, 4,6-dihydroxypyrimidine, uracil and barbituric acid were purchased from Aldrich and purified by recrystallization from appropriate solvents.

HPLC analysis

The HPLC analysis of incubation mixtures was performed as described previously[3]. The substrate concentration in the mixture was 0.2 mM and 25 mM potassium phosphate, pH=7.5 containing 0.1 mM EDTA was employed as a buffer. Registration of the oxidation products was carried out by UV detection at 254 nm (log ε for 4a: 3.90; 4b: 3.80; 4c: 3.89; 5b: 3.83; 5c: 3.83; 4,6-dihydroxypyrimidine: 3.89; barbituric acid: 4.24).

Synthesis of the uracil derivatives 4b-c and 5b-c with immobilized aldehyde oxidase

Rabbit liver aldehyde oxidase was immobilized by absorption onto DEAE Sepharose CL 6B as described before[3], applying 45 mg of aldehyde oxidase-containing protein per 3.5 ml packed Sepharose gel during immobilization. The immobilized enzyme preparation was packed in a column and washed with 10 mM potassium phosphate buffer, pH=7.5 (+ 0.1 mM EDTA) at 4°C. For each conversion 18 units of aldehyde oxidase were used (for definition, see *Kinetic assays*). The DEAE Sepharose was regenerated as described elsewhere[15].

A solution of 28 mg substrate in 500 ml 10 mM phosphate buffer, pH=7.5 (+ 0.1 mM EDTA) was pumped (45 ml/h) through the column at 4°C and recycled ounce or twice until the aldehyde oxidase activity was depleted. The formation of product was registrated at a suitable wavelength (see *Kinetic assays*). After evaporation of the collected effluent which was acidified to about pH=6.5, to dryness, the residue was purified by column chromatography (eluent chloroform-ethanol 9:1 (v/v)). The UV and mass spectra of the isolated products were identical with those of authentic materials. Yields, melting points and exact mass measurement data are collected in Table 6.2.

Table 6.2 Product yields, melting points and exact mass data of uracil derivatives 4b-c and 5b-c obtained by oxidation using immobilized aldehyde oxidase

Substrate	Product	Yield (%)	M.p.(0^oC)	Formula	Exact mass Exp.	Theor.
1b	4b	43	231-233[a]	$C_5H_6N_2O_2$	126.0429	126.0429
1c	4c	44	174-176[b]	$C_{11}H_{10}N_2O_2$	202.0747	202.0742
2b	4b	49	230-232[a]	$C_5H_6N_2O_2$	126.0428	126.0429
2c	4c	52	174-176[b]	$C_{11}H_{10}N_2O_2$	202.0742	202.0742
3b	5b	64	178-180[c]	$C_5H_6N_2O_2$	126.0429	126.0429
3c	5c	78	180-182[d]	$C_{11}H_{10}N_2O_2$	202.0742	202.0742

a. Lit.[9]: 232-233°C.
b. Lit.[13]: 173-174°C.
c. Lit.[9]: 179°C.
d. Authentic sample: 182-183°C.

Kinetic assays

The assay for aldehyde oxidase was carried out as described previously[3] and each assay was performed at least in duplicate. In the pH range studied (6.5-7.8) potassium phosphate buffers with an ionic strength, I = 0.05, containing 0.1 mM EDTA, were employed[16]. The wavelengths (λ in nm) and corresponding molar differential absorption coefficients (log $\Delta\varepsilon$) at pH=7.0 are: 1a-4a: 300 (3.71); 1b-4b: 302 (3.75); 1c-4c: 302 (3.76); 2a-4a: 258 (3.60); 2b-4b: 273 (3.72); 2c-4c: 246 (4.15); 3b-5b: 254 (3.68); 3c-5c: 258 (3.54). These $\Delta\varepsilon$ values were corrected for pH effects in the region pH > 7. For aldehyde oxidase one unit of enzyme activity is defined as the amount of enzyme which oxidized 1 µmol of 3-aminocarbonyl-1-methylpyridinium chloride per min at 25°C. The assay conditions were: 5 mM substrate in 50 mM potassium phosphate buffer, pH=7.8 (+ 0.1 mM EDTA) with the reaction being monitored at 292 nm (log $\Delta\varepsilon$=3.64). Kinetic data were calculated from Lineweaver-Burk plots[17].

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7 THE STABILITY OF FREE AND IMMOBILIZED ALDEHYDE OXIDASE

7.1 INTRODUCTION

The application of immobilized enzymes and cells in synthetic organic chemistry is a promising subject of research. Enzymes can often catalyze reactions, which are chemically not feasible or difficult to achieve, with a high specificity, making work-up of the reaction mixtures very easy. Furthermore, the reaction conditions required are usually very mild. The stability of free enzyme as well as the regenerability of reactive enzyme, however, have prohibited the use in organic chemistry for a long time. Immobilization of the enzyme can reduce these drawbacks. Immobilized enzymes often show an improved operational stability in comparison to their soluble counterparts, especially in the case of labile enzymes. In the laboratory of Organic Chemistry there is a strong interest in the chemistry of azaheterocycles. A study was initiated using these compounds as substrates in the reaction of immobilized hydroxylases. The hydroxylases which belong to the class of oxido-reductases, where chosen because of the broad substrate specificities towards these compounds. The oxidation of the six-membered heteroarenes by other methods is usually difficult, since the π-deficiency in the rings makes elektron donation to an oxidizing agent less easy or even impossible. The enzymes xanthine oxidase and xanthine dehydrogenase, isolated from different species, were succesfully employed in free or immobilized form for laboratory scale oxidations of azaheterocyclic compounds[1].
In recent papers we described the oxidation of N-alkyl and N-aryl azaheterocycles with immobilized rabbit liver aldehyde oxidase[2-5]. To date no report has appeared of our work on the immobilization of this latter enzyme; in this paper we present our first comprehensive study on this subject. Various immobilization procedures, including those employed in previous studies[2-5] will be discussed and tested for their applicability in organic synthesis.

7.2 RESULTS AND DISCUSSION

7.2.1 Purification of aldehyde oxidase

The procedure used to purify the enzyme has been described by Felsted *et al*[6]. As already reported in previous studies the partially purified aldehyde oxidase preparations were devoid of indigenous xanthine oxidase activity[2-5] and therefore

suitable for kinetic measurements. The purification procedure involves four steps: a heat treatment, ammonium sulfate precipitation, acetone fractionation and a hydroxylapatite column (used for preparations I-III). Preparations, which were not purified on the hydroxylapatite column, were usually employed for small scale conversions[3-5]; they also contained a low xanthine oxidase activity (preparation IV). Both types of preparations were tested for their applicability in organic synthesis.

In general the yield of the extracted total aldehyde oxidase activity, found after heat treatment, is considerably lower than the 365 units per 200 g of rabbit livers reported before[6]. The average value of eighteen purifications carried out, amounted to 143 ± 39 units of aldehyde oxidase per 200 g of livers. We usually started from frozen rather than fresh livers[6], and therefore the loss of aldehyde oxidase activity due to the freezing/thawing process was determined. A decrease in activity of about 18% was found compared to fresh livers. This decrease in activity was independent of the storage time of the frozen livers. Correction for the decrease gave an activity of 169 ± 39 units for fresh livers. The lower yield is partly due to the lesser sensitivity of the assay method employed here (about 9%). Since our goal is to apply aldehyde oxidase as a biocatalyst for preparative purposes, the lower yield of activity is a disadvantage but not prohibitive for its use. The specifications of the various aldehyde oxidase preparations used in this study are collected in Table 7.1. The degrees of purification are less than those reported[6], but the total yields of activity are comparable or significantly better. It is obvious that the application of a less purified preparation (IV) offers certain advantages with respect to both the yield of functional aldehyde oxidase and the time required for the purifica-

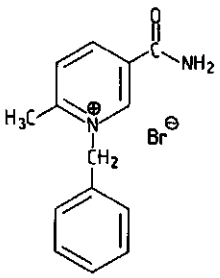
Table 7.1 Specifications of the applied aldehyde oxidase preparations

Preparation	Purification procedure (steps)	Specific activity (units/mg)	Purification[a,b] (fold)	Yield[b] (%)	A_{280}/A_{450}[c]
I	4	0.27	15.0 (35.6)	40 (42)	13.1
II	4	0.33	19.9 (35.6)	54 (42)	11.8
III	4	0.35	21.2 (35.6)	49 (42)	11.7
IV	3	0.10	6.4 (9.8)	76 (70)	17.3

a. Liver extract after heat treatment = 1.

b. The values in parentheses represent data reported previously[6].

c. Purity criterium. The lowest value reported[6] for highly purified enzyme is 5.2.



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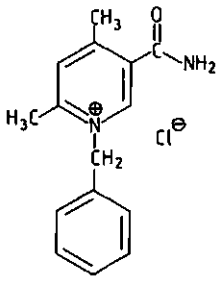

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tion procedure. Therefore, the use of this type of preparation (IV) is preferred under reaction conditions in which indigenous xanthine oxidase *i.* is inactive towards the substrate applied[3], *ii.* does not interfere with the aldehyde oxidase-mediated reaction[3,4], or *iii.* yields the same product as aldehyde oxidase[5]. During the course of our investigations we tried to improve the purification method employed by applying bioaffinity chromatography as described by Chu and Chaykin[7]. Their method has been advocated to give a high yield of an aldehyde oxidase preparation of high specific activity, completely devoid of any xanthine oxidase activity. In addition the complete procedure should take only one day. When we tested the described[7] affinity ligand 3-aminocarbonyl-1-benzyl-6-methyl-pyridinium bromide (1) with aldehyde oxidase, a similar competitive inhibition constant K_i was found as determined before (Table 7.2). HPLC analysis revealed, however, that the affinity ligand is slowly converted by the enzyme. Application of 3-aminocarbonyl-1-benzyl-4,6-dimethylpyridinium chloride (2) showed no oxidation by aldehyde oxidase, suggesting that the oxidation of the former compound

Table 7.2 Competitive inhibition constants K_i of potential bioaffinity ligands for aldehyde oxidase[a]

Ligand	K_i (μM)	
	pH = 7.8	pH = 9.0
1	25 ± 2[b]	14 ± 1
2	-	59 ± 4

a. In Tris-HCl (I=0.01), containing 0.1 mM EDTA.

b. K_i=30 μM; determined in 50 mM phosphate buffer[7].

probably occurs, quite unexpected, at C-4[2,3]. This result makes ligand 1 unattractive for application in bioaffinity chromatography. As shown in Table 7.2 the 4,6-dimethyl compound 2, although not oxidable by aldehyde oxidase, is not very suitable as ligand either, since its inhibition of the enzyme is even weaker. Therefore no further attempts to apply this type of purification procedure were made at this stage.

7.2.2 Immobilization of aldehyde oxidase onto n-alkylamine-substituted Sepharose and diethylaminoethyl Sepharose

A. Storage stability

For successful application in organic synthesis it is important that the immobilized enzyme possesses both a good storage stability and a long operational stability. The storage stability of aldehyde oxidase adsorbed to n-alkylamine-substituted Sepharose 4B[8,9] was tested in various buffers at pH=7.8 and pH=9.0 (Table 7.3); pH=7.8 represents the pH at which the aldehyde oxidase purification is carried out and pH=9.0 is about the upper chemical stability limit of the employed matrix material[10].

It is found that the stability is less at pH=7.8 than at pH=9.0 for both free and immobilized enzyme. Unexpectedly, free aldehyde oxidase is more stable on storage than the immobilized enzyme, irrespective of the buffer system used. The inactivation of immobilized aldehyde oxidase especially at 30°C occurs very

Table 7.3 Storage stability of aldehyde oxidase[a] in various buffers

buffer[b]	pH	Half life (days)			
		Free enzyme[c]		Immobilized enzyme[d]	
		4°C	30°C	4°C	30°C
50 mM phosphate	7.8	34	5.3	2.1	1
50 mM pyrophosphate	7.8	31	3	4.9	1
50 mM pyrophosphate	9.0	87	13.3	13.5	1
50 mM borate	9.0	108	13	6.8	0.6
Tris-HCl (I=0.01)	9.0	245	16.8	19.8	1.1

a. Preparation II.

b. All buffers contained 0.02% (w/v) NaN_3 and 0.1 mM EDTA.

c. Protein content was 0.5 $mg.ml^{-1}$.

d. Aldehyde oxidase adsorbed to n-octylamine-substituted Sepharose 4B. The protein content was 13.4 $mg.g^{-1}$ freeze-dried Sepharose 4B.

rapidly. The Tris-HCl buffer system is as yet preferred for storage of immobilized enzyme. Similar storage stabilities are found, when aldehyde oxidase is immobilized onto n-hexylamine-substituted Sepharose 4B or diethylaminoethyl (DEAE) Sepharose CL 6B, using the same amount of protein per gram of matrix material, and stored in Tris-HCl (I=0.01), pH=9.0. The results indicate that studies on the kinetics of immobilized aldehyde oxidase with various (new) substrates are very restricted. It is evident that for synthetic applications the immobilization procedure must be performed directly prior to use in order to obtain maximal substrate conversion.
Compared to bovine milk xanthine oxidase and chicken liver xanthine dehydrogenase immobilized to the same material, the results are completely opposite, since these enzymes show a substantial increase in storage stability upon immobilization[9,11].

B. Operational stability

A second important aspect of free and immobilized enzyme concerns the operational stability. The operational stability and productivity of several aldehyde oxidase/n-octylamine Sepharose 4B preparations are presented in Table 7.4. As observed for bovine milk xanthine oxidase immobilization of aldehyde oxidase to this matrix increases the operational stability considerably in comparison to free enzyme incubated with a 0.5 mM substrate solution (protein content 0.2 $mg.ml^{-1}$). Rapid inactivation of free aldehyde oxidase upon incubation with this substrate was also observed by Coughlan *et al.*[12]. The fast inactivation is probably not due to the loss of flavin, because incubation of partially inactive enzyme with FAD under proper conditions[13,14] did not restore activity. In the first half life the immobilized enzyme yields 17 μmoles of product per unit of immobilized activity, which corresponds to about 5 μmoles of product per milligram of immobilized protein (Table 7.4). Compared to xanthine oxidase adsorbed to this matrix ($t_{\frac{1}{2}}$ = 0.8 days, productivity = 29 μmoles per mg protein)[15] both the half life and productivity of the immobilized aldehyde oxidase preparation are very poor. During operation no significant leaching of protein from the support occurred.
It has been well established that hydrogen peroxide and/or superoxide is produced during the catalysis with hydroxylases. These compounds can exert a directly or indirectly damaging effect on the enzymes themselves[16,17]. We therefore determined the influence of catalase and superoxide dismutase on the half life and the corresponding productivity of immobilized aldehyde oxidase. Coimmobilization with either catalase or superoxide dismutase shows an equally positive effect on

Table 7.4 Operational stability and productivity of various aldehyde oxidase/Sepharose 4B preparations[a] at pH=9.0 and 4°C

Preparation[b]	Half life $t_{\frac{1}{2}}$[c] (min)	Productivity in $t_{\frac{1}{2}}$[c] (μmol/unit)
free AO	<15	-
adsorbed AO	290	17[d]
coadsorbed AO and CAT	365	20.5
coadsorbed AO and SOD	360	19.5
coadsorbed AO and CAT and SOD	390	23.5
coadsorbed AO and BSA	305	18

a. Preparation I immobilized onto n-octylamine-substituted Sepharose 4B.

b. Amount of protein offered per gram of freeze-dried CNBr-activated Sepharose 4B during immobilization: 7.5 mg aldehyde oxidase (AO), 2 mg catalase (CAT; 3000 units.mg^{-1}), 2 mg superoxide dismutase (SOD; 2800 units.mg^{-1}), 4 mg bovine serum albumin (BSA).

c. Average of two determinations.

d. All aldehyde oxidase activity and 95% of the offered amount of protein was adsorbed to the carrier for this enzyme preparation. The productivity value is therefore identical to 17 x 0.27 x 1.05 ≈ 5 μmol/mg protein.

the half life, whereas the productivity increased more by coimmobilization of catalase. This result is in full agreement with the limited stability of immobilized superoxide dismutase found under turnover conditions in a small xanthine oxidase/superoxide dismutase reactor[9]. When both enzymes are immobilized together with aldehyde oxidase, both the half life and productivity improve even more. In a control experiment with coimmobilized bovine serum albumin, the stabilization effect of the inert protein is substantially, less and therefore, the protective effect of catalase and superoxide dismutase has to be attributed to their mode of operation. Moreover, the location of both enzymes close to aldehyde oxidase is very important for obtaining a stabilizing effect on aldehyde oxidase during catalysis, because *free* enzyme, upon incubation with substrate, was found to remain unprotected against inactivation by the inclusion of soluble superoxide dismutase and catalase[12].

Despite the improved stability of immobilized enzyme, when catalase and/or superoxide dismutase are included, the effect is still rather limited and requires the use of the expensive and relatively unstable superoxide dismutase, to obtain optimal results. Furthermore, the leakage of catalase and superoxide dismutase from the column during operation is a substantial drawback[9]. These

aspects make the latter preparations less suitable for preparative scale conversions.

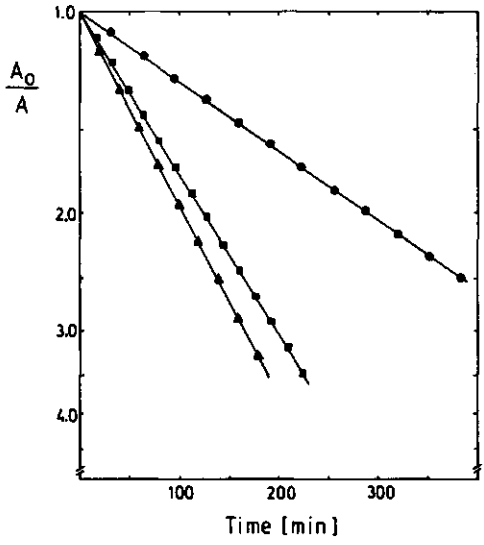

Figure 7.1 Effect of the rate of oxidation on the operational stability of aldehyde oxidase adsorbed to n-octylamine-substituted Sepharose 4B at pH = 9.0. The ratio of the conversion at t=o (A_o) and t(A) is plotted. Per gram dry support 7.5 mg of protein (preparation I) was applied. (●--●) 0.5 mM substrate solution, 4°C; (■--■) 0.5 mM substrate solution, 25°C; (▲--▲) 5mM substrate solution, 4°C.

The inactivation of immobilized aldehyde oxidase during continuous turnover is dependent on the rate of oxidation, which is demonstrated by increasing the reaction temperature or the substrate concentration. As shown in Figure 7.1 the operational half lives drop more than twofold compared to that of a preparation tested under standard conditions (see Experimental Section). The corresponding productivities in the first half life time are found to be 10 µmoles per unit of immobilized activity at 25°C and 4.5 µmoles per unit of immobilized activity, when a substrate solution of 5 mM is applied.

C. Effect of the protein load on the operational stability

Another approach to improve the efficiency of aldehyde oxidase/n-octylamine Sepharose 4B preparations, consisted of increasing the load of aldehyde oxidase preparation per gram of matrix material. In addition we also tested this method for the analogous supports n-hexylamine-substituted Sepharose 4B and DEAE Sepharose CL 6B. The latter matrix possesses a hydrophilic nature and adsorbs proteins mainly by ionic binding[18], whereas n-hexyl- and n-octylamine-substituted Sepharose 4B combine both ionic binding and hydrophobic properties[8]. Moreover, all these matrices can be regenerated in principle after depletion of the aldehyde oxidase activity[18-20].

The concentration dependence of the protein adsorption and aldehyde oxidase retention is depicted in Figure 7.2. It becomes evident from the protein adsorption isotherms (Figure 7.2a) that more protein can be loaded on the n-alkylamine-substituted matrices as compared to the hydrophilic DEAE Sepharose, although the difference in protein loading capacity between n-hexylamine Sepharose 4B and DEAE Sepharose CL 6B is rather small. The units of aldehyde oxidase activity bound decrease rapidly for the n-hexylamine-substituted matrix at higher protein concentrations, whereas both n-octylamine-substituted Sepharose and DEAE Sepharose adsorb the enzyme proportionally up to about 60 mg of protein offered per gram matrix (Figure 7.2b).

The effect of the protein load on the operational half life of these immobilized preparations is shown in Figure 7.3. The operational half life of aldehyde oxidase adsorbed to n-alkylamine-substituted Sepharose is affected differently than that of enzyme immobilized on DEAE Sepharose. Linear relationships indicating that the half life is proportional to the amount of protein bound are established in the case of the former matrices, while a maximum operational stability is found with the latter.

The productivity per unit immobilized aldehyde oxidase in the first half life exhibits a similar behaviour as found for the operational half life: it reaches a maximum for aldehyde oxidase/DEAE Sepharose preparations at increasing protein load, but is about proportional for both other immobilized preparations.

Since saturation for DEAE Sepharose is attained at about 35 mg aldehyde oxidase preparation adsorbed per gram of matrix (Figure 7.2a), bovine serum albumin was coimmobilized to obtain a higher protein load. It is clear from the productivity curve of aldehyde oxidase/DEAE Sepharose preparations the protein load has hardly any positive effect on the productivity above 20 mg of protein adsorbed per gram carrier (Figure 7.3b). The difference between both types of matrices in

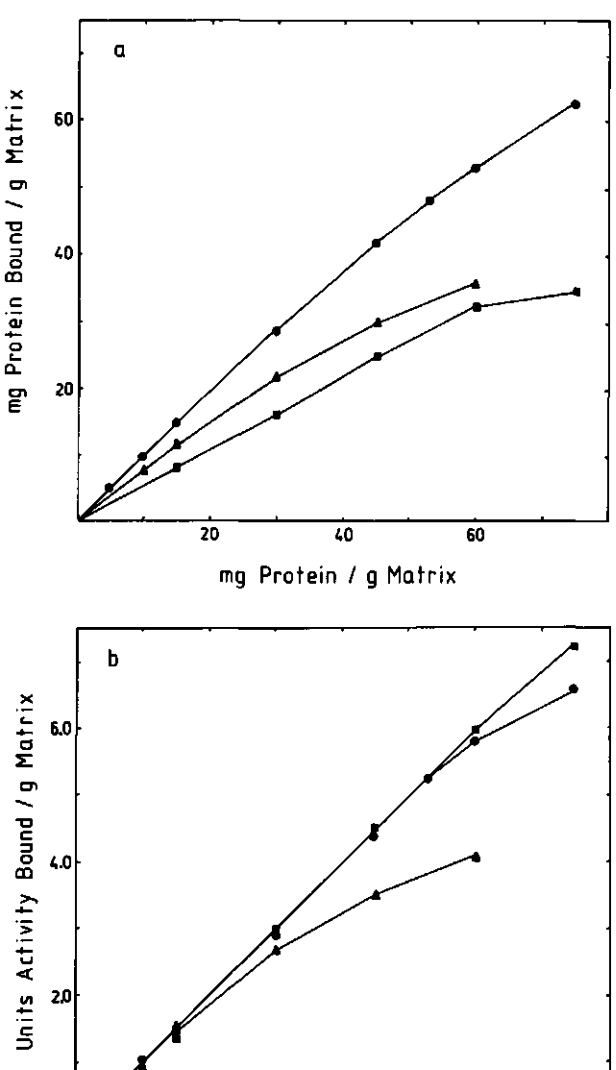

Figure 7.2 *(a) Protein adsorption isotherms of an aldehyde oxidase preparation (IV) to various Sepharose adsorbents at 4°C and pH=9.0. (●--●) n-octylamine-substituted Sepharose 4B (degree of substitution: 5.1 µmol Ponceau S/g dry support); (▲--▲) n-hexylamine-substituted Sepharose 4B (degree of substitution: 5.3 µmol Ponceau S/g dry support); (■--■) DEAE Sepharose CL 6B.*
(b) Aldehyde oxidase adsorption isotherms for enzyme preparation IV at 4°C and pH=9.0. Symbols, see (a).

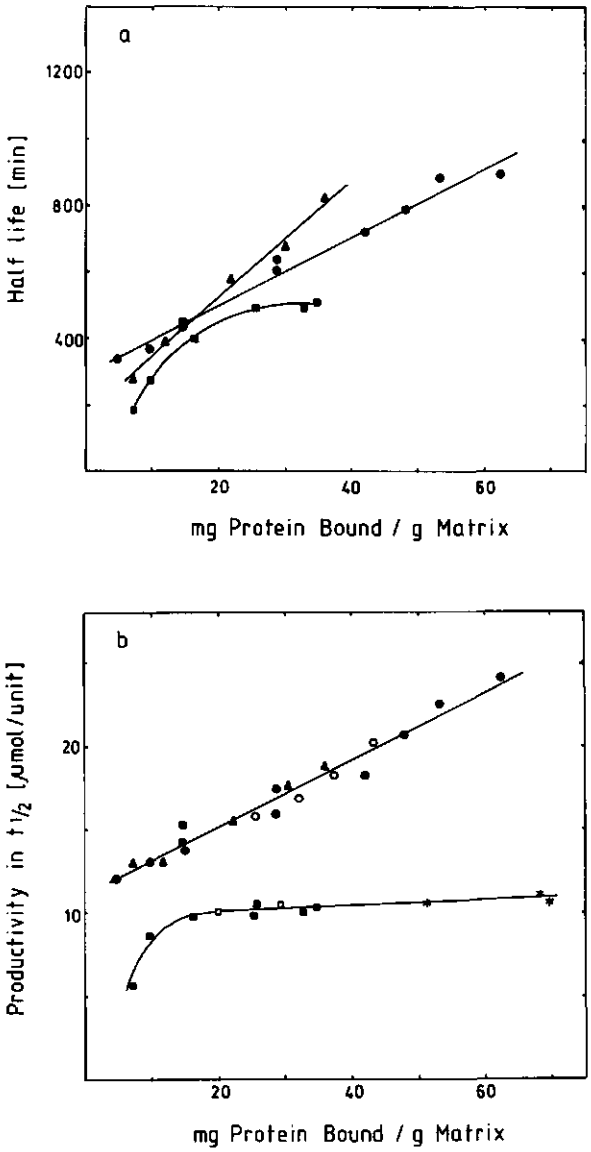

Figure 7.3 (a) Operational half life of aldehyde oxidase immobilized to various Sepharose adsorbents at 4°C. Feed: 1 mM substrate, pH=7.8. For symbols, see Fig. 7.2.
(b) Productivity in the first half life of aldehyde oxidase immobilized to various Sepharose adsorbents at 4°C. (o--o) enzyme preparation III adsorbed to n-octylamine-substituted Sepharose 4B; (□--□) enzyme preparation III adsorbed to DEAE Sepharose CL 6B; (--*) enzyme preparation IV coadsorbed with bovine serum albumin to DEAE Sepharose CL 6B. For other symbols, see Fig. 7.2.*

respect to the productivity of aldehyde oxidase, can be explained by the occurrence of hydrophobic proteins in the enzyme preparation, which stabilize the aldehyde oxidase activity during catalysis, and which are not adsorbed to DEAE Sepharose. No significant disparities are found between the productivities of the n-hexylamine and n-octylamine Sepharose 4B/aldehyde oxidase preparations at varying protein concentrations. Application of enzyme preparations of different purity, *i.e.* preparations III and IV, does not result in a significant effect on the productivity.
Testing of regenerated n-octylamine-substituted Sepharose 4B showed that the capacity of the support gradually decreases after repeated use, while the productivity per unit of immobilized aldehyde oxidase remained equal to that of enzyme adsorbed to fresh matrix material.

7.2.3 Immobilization of aldehyde oxidase to other supports

Aldehyde oxidase was also immobilized to other matrices and/or by other methods. Some of the results are recorded in Table 7.5.
In gelatin entrapped aldehyde oxidase crosslinked with glutaraldehyde has a longer half life than enzyme adsorbed to n-octylamine Sepharose 4B, but the productivity is extremely low. We established that when gelatin was replaced by bovine serum albumin about 50% of the aldehyde oxidase activity was lost during the crosslinking procedure with glutaraldehyde at the protein concentration applied (40 $mg.ml^{-1}$). Since alifatic aldehydes usually are substrates for aldehyde oxidase[21], it is very likely that the inactivation occurred by a chemical reaction in the active centre of the enzyme. Moreover, the stabilization by gelatin is negligible and correction for the initial loss of activity due to crosslinkage would still yield a low productivity value of 7 μmoles per immobilized unit of aldehyde oxidase. This result is completely converse to that reported for bovine milk xanthine oxidase, which is excellently stabilized by the gelatin matrix during turnover[15].
Covalently bound aldehyde oxidase on silica possesses a very short half life and the productivity has only slightly improved in comparison to the gelatin preparation. Furthermore, only about 60% of the offered aldehyde oxidase activity is bound during immobilization.
Adsorption to controlled-pore titania results in a preparation with an extremely short half life, although the productivity is equal to that of the aldehyde oxidase/n-octylamine Sepharose 4B preparation (Table 7.4). The binding capacity is low for the titanium material, since merely about 40% of the aldehyde oxidase activity is adsorbed. Another disadvantage is the abrasion of matrix material

Table 7.5 Operational stability and productivity of various immobilized aldehyde oxidase (AO) preparations at pH=9.0 and 4°C

Preparation[a]	Half life $t_{\frac{1}{2}}$[b] (min)	Productivity in $t_{\frac{1}{2}}$[b] (μmol/unit)
Gelatin entrapped and glutaraldehyde crosslinked AO	540	3.5
Covalently bound AO on silica	185	5.5
Titania adsorbed AO	95	17
n-Octyl Sepharose CL 4B adsorbed AO	310	12.5
Phenyl Sepharose CL 4B adsorbed AO	295	11

a. Fifteen mg of protein of enzyme preparation III was applied during immobilization per gram of support or per 3.5 ml of packed Sepharose gel.

b. Average of two determinations.

during operation, despite of intensive washings with buffers of high ionic strength prior to the run.

The pure hydrophobic supports n-octyl and phenyl Sepharose CL 4B both have a low adsorptive capacity for aldehyde oxidase. Only 18% and 15% of the offered activity are retained on these materials, respectively. The half lives of the preparations are comparable to that established for the aldehyde oxidase/n-octylamine Sepharose 4B preparation (Table 7.4), while the corresponding productivities are substantially less. Another drawback of this type of matrices is the small but continuous leakage of protein from the column when no high salt concentration is applied[20].

Various other supports and/or immobilization methods have been tested, like covalent coupling to CNBr-activated Sepharose 4B, to Enzacryl AA, to carboxymethylcellulose and adsorption to activated carbon. The obtained preparations were devoid of, or showed hardly any aldehyde oxidase activity.

7.3 EXPERIMENTAL SECTION

Materials

Fresh rabbit livers were obtained from Wilco bv (Culemborg, The Netherlands) and used immediately or stored frozen at -25°C until required. Catalase from beef liver (E.C. 1.11.1.6) was purchased from Boehringer and bovine erythrocyte superoxide dismutase (E.C. 1.15.1.1) from Sigma. Bovine serum albumin was from

Boehringer and hydroxylapatite (Bio-Gel HT) from Bio-Rad. Freeze-dried CNBr-activated Sepharose 4B, Phenyl Sepharose CL 4B, Octyl Sepharose CL 4B and DEAE Sepharose CL 6B were obtained from Pharmacia Fine Chemicals. Enzacryl AA was from Koch-Light Laboratories, controlled-pore titania (500Å, 45-60 mesh) from Corning, activated carbon (28 mesh) from Pierce Inorganics, carboxymethylcellulose hydrazide from Sigma and silica coated with N-hydroxysuccinimide-activated carboxylgroups on C_5-spacers from Diosynth.
3-Aminocarbonyl-1-methylpyridinium chloride, 3-aminocarbonyl-1-benzyl-6-methylpyridinium bromide, 3-aminocarbonyl-1-benzyl-4,6-dimethylpyridinium chloride were synthesized according to described methods[2,3] and were analytically pure. Glutaraldehyde (Merck), gelatin (Merck), Ponceau S (Searle), n-hexylamine (Fluka), n-octylamine (Merck), Folin reagent (Merck) and all other materials employed, were at least reagent grade.

Purification of aldehyde oxidase

Aldehyde oxidase (E.C. 1.2.3.1) was purified from rabbit livers according to the procedure of Felsted *et al.*[6], by performing three (preparation IV, Table 7.1) or four (preparations I-III, Table 7.1) of the six purification steps. Hydroxylapatite was employed instead of calcium phosphate gel and the enzyme was eluted from this material with a linear gradient of 0-200 mM potassium buffer, pH=7.8 containing 0.1 mM EDTA. Fractions with main aldehyde oxidase activity were combined and the protein was precipitated by careful addition of ammonium sulphate (0.60 saturation). After centrifugation the material was resuspended in Tris-HCl (I=0.01), pH=7.8 containing 0.1 mM EDTA and stored at -25°C until use. The protein material acquired by the three step-procedure was resuspended in the same buffer directly after acetone fractionation, the suspension was clarified by centrifugation, dialyzed and stored under identical conditions.

HPLC analysis

The HPLC analysis of incubation mixtures of the bioaffinity ligands with aldehyde oxidase (preparation I) was carried out as described previously[2], using a Varian 5000 instrument equipped with a Micro Pak MCH-10 column (30 x 0.4 cm), a Schoeffel GM-770 monochromator and an SF-770 spectroflowmonitor. Operating conditions were Δp=17.2 MPa, eluent water/methanol 60:40 (v/v) and flowrate 2.0 ml/min. Registration of oxidation products occurred by UV detection at 254 nm[3].

Immobilization of aldehyde oxidase

Covalent coupling to Sepharose 4B occurred analogously to a procedure described before[9]. 7.5 mg of the enzyme preparation III were applied per gram of CNBr-activated Sepharose 4B.

For the adsorption to Sepharose matrices one gram (based on dry weight of CNBr-activated Sepharose 4B) of n-alkylamine-substituted Sepharose 4B[8] or 3.5 ml packed gel of preswollen material, corresponding to about one gram of dry material[18] (in the case of DEAE Sepharose and the hydrophobic Sepharoses) was washed with 30 mM borate buffer, pH=9.0 (0.1 mM EDTA) on a sintered-glass funnel with suction. The adsorbent was then transferred to a round bottom flask and 30 ml of a protein solution in borate buffer added. Adsorption was accomplished by rotation of the mixture during 5 hours at 4°C. The immobilizate was washed with the same buffer several times. The degree of substitution of Sepharose 4B with n-alkylamine was determined by the method based on the irreversible binding of Ponceau S to this support material at low ionic strength[22].

Entrapment in gelatin, followed by crosslinkage with glutaraldehyde[15] and coupling to carboxymethylcellulose hydrazide[23] was carried out according to described procedures.

Adsorption to activated carbon was performed after cleaning of the material by the acidic pretreatment as described by Cho and Bailey[24]. One gram of cleaned material was rotated after deaeration together with 30 ml of a protein solution (0.5 $mg.ml^{-1}$) in 30 mM borate buffer, pH=9.0 containing 0.1 mM EDTA at 4°C. After 5 hours the adsorption procedure was stopped and the activated carbon washed thoroughly with the same buffer.

Controlled-pore titania was cleaned prior to use to remove easily grindable particles by rotating the deaerated material in a round bottom flask in 25 ml of 0.1 M sodium citrate buffer, pH=5.5. After 30 minutes the supernatant was decanted and the procedure was repeated three times. The material was then washed with citrate buffer on a sintered-glass funnel, packed into a column and again washed thoroughly with 30 mM borate buffer, pH=9.0 (0.1 mM EDTA). The enzyme solution in borate buffer was loaded onto the column and slowly soaked in until about one void volume had eluted from the column. After standing overnight at 4°C the column was washed thoroughly with borate buffer.

Covalent coupling to Enzacryl AA was essentially identical to the method employed for coupling to carboxymethylcellulose[23]. The coupling time was extended to 24 hours. The immobilizate was stirred magnetically in an ice-cold solution of 0.01% phenol in 10% sodium acetate for 15 minutes before washing of the material.

For coupling to silica coated with N-hydroxysuccinimide-activated carboxyl groups on C_5-spacers, the dry beads were suspended in 0.1 M bicarbonate buffer, pH=9.0 and the suspension transferred into a round bottom flask which was attached to a vacuum evaporator. The beads were carefully deaerated under rotation and the enzyme preparation was then added to the suspension. The reaction was accomplished in 3 hours at room temperature, while the pH was measured and adjusted at intervals of 30 minutes. After decantation of the suspension the buffer was replaced by a solution of 0.1 M ethanolamine, pH=8.5. The reaction was complete after 2 hours and during this period the pH was adjusted regularly (*e.g.* every 15 minutes). The immobilizate was next washed on a sintered-glass funnel with coupling buffer, 0.1 M Tris buffer (pH=8.0 containing 2 M NaCl) and 0.1 M acetate buffer (pH=4.0 containing 2 M NaCl), respectively.

Enzyme stability

The storage stability of free and immobilized enzyme was determined by incubation of a series of enzyme samples in the buffer studied, containing 0.02% (w/v) sodium azide at two different temperatures. The activity of the samples was assayed as a function of time.

For the determination of the operational stability, 1 g of the freshly prepared immobilizate (for the gelatin preparation 5 g), based on the dry weight of the carrier, was packed into a column (i.d. 0.5 or 2.5 cm, the latter only being employed for the gelatin preparation) and washed with Tris-HCl (I=0.05), pH=7.8 or Tris-HCl (I=0.01), pH=9.0, both containing 0.1 mM EDTA. The buffer used is specified by the pH given in the Figures and Tables.

After the washing procedure (A_{280}=0) an 0.5 mM solution of 3-aminocarbonyl-1-methylpyridinium chloride (unless specified otherwise) in the corresponding buffer was pumped at a constant flow rate (15 $ml.h^{-1}$) through the column and the absorbance of the eluate at 300 nm, *i.e.* the formation of product (log $\Delta\varepsilon$= 3.62)[6], was measured as a function of time. The columns were run at 4°C (unless specified otherwise), avoiding normal levels of room light.

Protein determination

All protein determinations were performed with the modified Lowry microprocedure[25], using bovine serum albumin as standard. This method was also suitable for determination of protein immobilized to n-alkylamine-substituted Sepharose 4B. The samples were incubated with the Lowry reagents at room temperature in the dark and the mixtures were shaken occasionally. After about 24 hours the mixtures were filtered and the absorbance at 700 nm measured. The assays were

performed in duplicate. If necessary the washings were concentrated and desalted by dialysis prior to the determination.

Enzyme-activity assays

In the standard activity assay for soluble aldehyde oxidase the reaction mixture contained 5 mM 3-aminocarbonyl-1-methylpyridinium chloride as a substrate in 50 mM potassium phosphate buffer, pH=7.8 (+ 0.1 mM EDTA) and a suitable amount of enzyme in a final volume of 2.5 ml. The temperature of the assay mixture was maintained at 25°C. The oxidation of substrate was monitored at 292 nm (log $\Delta\varepsilon$= 3.64) using an Aminco DW-2a UV-VIS spectrophotometer, which operated in split-beam mode. The rate of oxidation was determined from the initial slope of the absorbance versus reaction time curve, representing the rate of the formation of product. For aldehyde oxidase one unit of enzyme activity is defined as the amount of enzyme which oxidized 1 µmol of the substrate per minute at 25°C.
The activity assays for free aldehyde oxidase in other buffer systems for determination of storage stabilities were carried out analogously in the corresponding buffers.
The assay of the immobilized enzyme was performed under the same conditions as for free enzyme, but the sample volume was always 0.5 ml taken from a suitable diluted homogenous suspension with an Eppendorf pipet (analogous to the procedure developed by Mort *et al.*[26]). The mixture in the cuvet was stirred rapidly during the assays so that a homogeneous suspension was obtained and external diffusion limitation (*i.e.* diffusion limitation of substrate from the bulk into the gel) was minimized.
Activity assays were always performed in duplicate for free aldehyde oxidase and in triplicate for immobilized enzyme. The catalase activity assay was essentially that described by Beers and Sizer[27] and superoxide dismutase activity was determined by the method of McCord and Fridovich[28].
The inhibition of the oxidation of 3-aminocarbonyl-1-methylpyridinium chloride by the bioaffinity ligands was determined under the same assay conditions as described before[2]. Inhibition constants were calculated from Lineweaver-Burk[29] plots and replots.

Acknowledgement

We are indebted to Mr.D.J.Buurman for the synthesis of both bioaffinity ligands.

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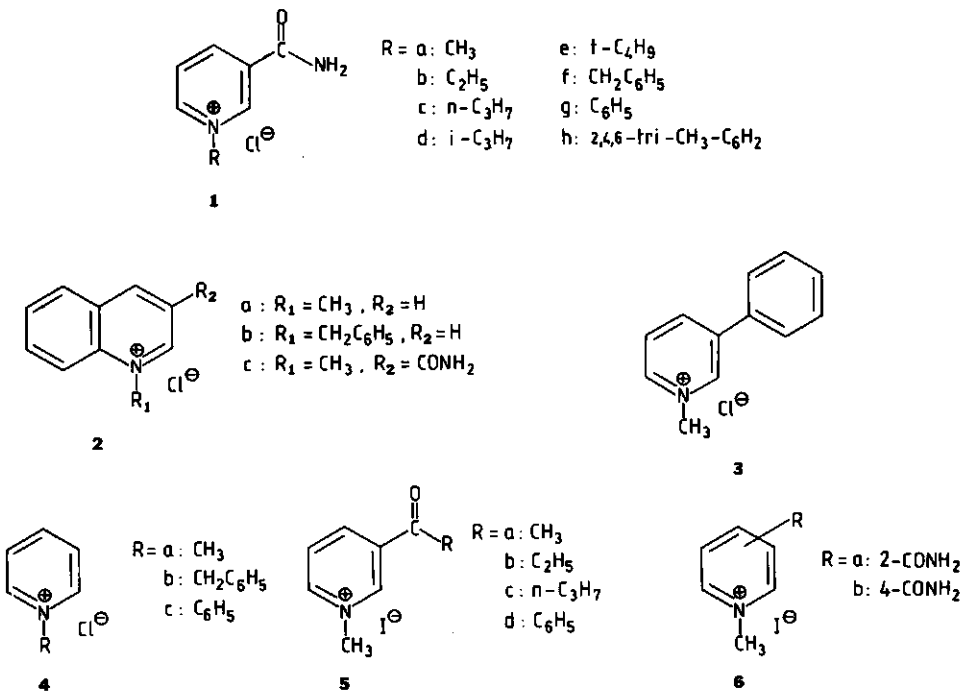
8 GENERAL DISCUSSION

In this thesis interesting results are presented on the catalytic activity of rabbit liver aldehyde oxidase towards N-alkyl(aryl) substituted pyridinium, quinolinium and pyrimidine compounds, in which the alkyl(aryl) substituents differ in size and/or electronic effects. Comparison of the substrate specificities of aldehyde oxidase and bovine milk xanthine oxidase shows quite distinct differences for both enzymes with regard to N-alkyl(aryl)azinium compounds (Scheme 8.1). A survey of these substrate specificities based on data presented in the previous chapters and supplemented with some unpublished results is given in Table 8.I. It is obvious that aldehyde oxidase possesses a broader specificity towards these compounds than xanthine oxidase.



Scheme 8.1

Moreover, xanthine oxidase only significantly converts N-alkyl(aryl)azinium salts at pH $\geq$ 9.0[1], whereas aldehyde oxidase can operate in a wider pH-range (6.5-10; chapters 3 and 5). The variation in size and thus steric conformation of the N-substituent restricts the oxidation of these compounds by xanthine oxidase more than by aldehyde oxidase. Substitution of the pyridinium or quinolinium ring at other positions than at the ring nitrogen atom affects the substrate specificity of xanthine oxidase to a much lesser degree[1,2].

Table 8.1 Comparison of the substrate specificities of aldehyde oxidase (AO) and xanthine oxidase (XO) for various N-alkyl(aryl)azinium salts

Compound	Oxidation		Site of oxidation[a]	
	AO	XO	AO	XO
1a	+	+[b]	6	6[b]
1b	+	+	6	6
1c	+	-	6	-
1d	+	-	6(4)	-
1e	+	-	4	-
1f	+	-	6	-
1g	+	+	6(4)	4(6)
1h	+	-	4	-
2a	+	+[b]	2(4)	2[b]
2b	+	-	4(2)	-
2c	+	+	4	4
3	+	-[c]	6	-[c]
4c	+	+[b]	4	4[b]

a. Site of oxidation of minor product in parentheses.

b. In agreement with reference 1.

c. Reference 2.

The interaction of a (proton-donating) active site species in xanthine oxidase with these substrates[1,2] exhibits a less pronounced effect on the site of oxidation than that established for aldehyde oxidase (chapter 5). Oxidation of both the 1-phenylpyridinium salt and the corresponding 3-aminocarbonyl analogue by xanthine oxidase occurs exclusively or predominantly at C-4, whereas with aldehyde oxidase the predominant (exclusive) site of oxidation shifts from C-4 to C-6 upon the introduction of a 3-$CONH_2$ group. Obviously the relative location of this interactive species with regard to a hydrophobic region, which is very likely an important site in determining the binding of substrates in the active centre[3,4](chapter 2) and the catalytic nucleophilic group (chapter 3) is different in both enzymes.

As an extension of the work on the 1-alkyl(aryl)-3-aminocarbonylpyridinium salts we also investigated the oxidation of pyridinium derivatives by aldehyde oxidase in dependence of the presence, the position or the nature of the C-substituent. Semi-quantitative results of these reactions are collected in Table 8.2.

Table 8.2 Oxidation of various pyridinium compounds by aldehyde oxidase at pH = 7.8

Compound	Oxidation rate[a]	Site of oxidation
1a	+++	6
3	+	6
4a	-	-
4b	-	-
4c	+	4
5a	++++	6
5b	++++	6
5c	++++	6
5d	++++	6
6a	-	-
6b	+	b

a. Rate of oxidation: ++++ = fast, +++ = moderate, ++ = slow, + = very slow, - = no oxidation.

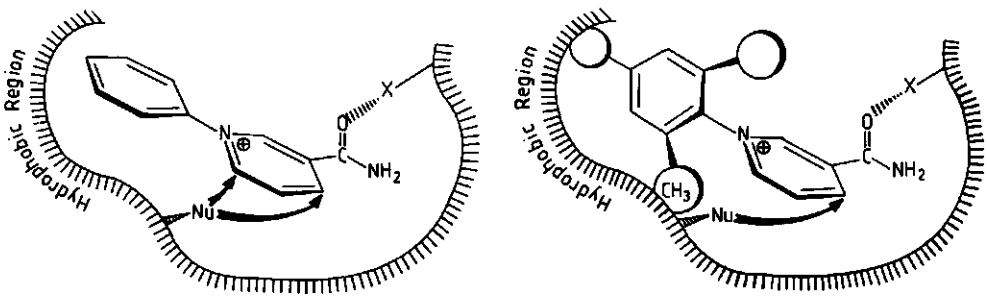
b. Not determined.

1-Methylpyridinium derivatives which contain a 3-alkanoyl(benzoyl) substituent (5a-d) are found to be oxidized at a higher rate than 3-aminocarbonyl-1-methylpyridinium chloride in 1a. Oxidation of these compounds occurs exclusively at C-6, showing that the enzyme has a great steric "tolerance" at this site (C-3). The presence of a phenyl group instead of a benzoyl group at C-3 (*i.e.* in compound 3) results in a low rate of oxidation by aldehyde oxidase, indicating the importance of the carbonyl moiety.

As mentioned before this moiety, most probably orientates the substrate molecule in the catalytic centre of the enzyme through interaction with a (proton-donating) active site species. In the case of substrates 3 and 5d the accessibility of C-6 for the enzymic nucleophile evidently is much better for the latter compound, due to this interaction.

Further proof for this suggestion is obtained from the reaction of pyridinium salts 4 containing no substituent at C-3. Compounds 4a and 4b are not converted at all and with the 1-phenyl analogue 4c oxidation takes place at a very low rate exclusively at C-4, which is comparable to the oxidation of 1-(4'-pyridyl)pyridinium chloride by this enzyme[5].

Oxidation of 1-methylpyridinium compounds with an aminocarbonyl substituent at C-2 or C-4 is not feasible or occurs very slowly (Table 8.2), indicating that these compounds are not oriented in the right fashion in the catalytic centre due to interaction of the carbonyl moiety with an active site species.



I II

Figure 8.1 Schematic representation of the orientation of two 3-aminocarbonyl-1-arylpyridinium substrates in the active centre of aldehyde oxidase during catalysis. Nu: enzymic nucleophilic species; X : interactive species.

A very simplified schematic representation of the orientation of two different azinium substrates in the catalytic centre of aldehyde oxidase is illustrated in Figure 8.1, based on the three important features of the active site suggested in this study, viz. the presence of a hydrophobic region (chapter 2), a rate-limiting nucleophilic attack (chapter 3) and the interaction with a (proton-donating) active site species (chapter 5). For the sake of simplicity only two pyridinium substrates are depicted, which differ in their steric conformation due to the presence of methyl substituents. In the oxidation of 3-aminocarbonyl-1-phenylpyridinium chloride the pyridinium ring is rotated in a position which brings about a favourable interaction of the carbonyl moiety of the 3-aminocarbonyl group with the active site species (I). The phenyl substituent interacts with the hydrophobic region in the vicinity of the catalytic site. In this orientation C-6 is much better accessible for nucleophilic attack than C-4, as has been established from the respective maximum rates of oxidation (chapter 3). The conformation of the aminocarbonyl group in the substrate and in the intermediate formed after nucleophilic attack at C-4 can have an effect on the overall orientation during catalysis as well[6-8].

The substrate 3-aminocarbonyl-1-(2,4,6-trimethylphenyl)pyridinium chloride has a steric conformation in which both ring planes are nearly perpendicular, because of the interaction of the *ortho*-methyl groups. This conformation will be at least partly retained in the active centre during catalysis. The approach of the nucleophilic species at C-6 (or C-2) is now severely hindered by the *ortho*-methyl groups and therefore, the oxidation appears to occur exclusively at C-4 (II). Because of the difference in hydrophobicity of the N-substituent it can be expected that the interaction of this substituent with the hydrophobic region of the enzyme will be slightly different, resulting in another orientation of the substrate molecule. This may lead to a greater accessibility of the C-4 site for the enzymic nucleophilic species and correspondingly the maximum rate of oxidation is much higher than that for oxidation of 3-aminocarbonyl-1-phenylpyridinium chloride at this site (chapter 3).

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SUMMARY

Aldehyde oxidase isolated from rabbit liver is studied in this thesis with regard to its application in organic synthesis. The enzyme has a broad substrate specificity towards azaheterocycles and therefore offers great potential for profitable use.

The oxidation of *1-alkyl(aryl)-3-aminocarbonylpyridinium chlorides* by aldehyde oxidase shows that reaction can occur in principle at two different positions in the pyridinium ring. Only the 1-alkyl-1,6-dihydro-6-oxo-3-pyridinecarboxamides are obtained with the 1-methyl, 1-ethyl and 1-*n*-propyl derivatives. The corresponding 4-oxo compound is found as sole product with the 1-*t*-butyl analogue, while the 1-*i*-propyl derivative yields a mixture of 4- and 6-oxo compounds. Evidence for the presence of a hydrophobic region in the vicinity of the enzymic active site has been acquired from the kinetic data of the oxidation reactions (chapter 2). Oxidation of the 1-aryl analogues results predominantly in 1-aryl-1,6-dihydro-6-oxo-3-pyridinecarboxamides, together with the corresponding 4-oxo compounds as minor products. In general the site of oxidation is determined by steric factors, although the maximum rate of oxidation by aldehyde oxidase is very sensitive to electronic effects. A more electron-withdrawing aryl substituent increases the reaction rate for oxidation at C-6. Consequently a positive ρ-value of about 3.6 is calculated for free aldehyde oxidase, indicating that a nucleophilic attack is the rate-limiting step in the oxidation mechanism of these azinium compounds. The oxidation of the 1-aryl derivatives by bovine milk xanthine oxidase yields mainly 4-oxo products. The maximum rate of oxidation with xanthine oxidase is only slightly affected by the nature of the aryl substituent (chapter 3).

1-Alkyl(aryl)quinolinium chlorides are also oxidized by aldehyde oxidase essentially at two positions, C-2 and C-4. The site and the maximum rate of oxidation are dependent on the size and the steric conformation of the N-substituent. The presence of an aminocarbonyl group at C-3 directs the oxidation completely to C-4, irrespective of the size of the N-substituent (chapter 5).

A comparison is made between the site(s) of oxidation of several azinium compounds by aldehyde oxidase and the covalent amination pattern in liquid ammonia. It is shown that covalent amination is particularly valuable as a model reaction with

those substrates in which the enzyme reaction is predominantly controlled by steric factors. Thus the oxidation positions of the 1-alkyl-3-aminocarbonylpyridinium chlorides are predicted quite accurately by the model reaction (chapters 4 and 5).

N-Methyl and N-benzyl derivatives of *2(1H)- and 4(3H)-pyrimidinone* (at N-1 or N-3) are oxidized at the same site by aldehyde oxidase. Despite the difference in steric size only one type of product has been obtained from all substrates studied, viz. the corresponding N-1 or N-3 substituted uracil. The maximum rates of oxidation with this enzyme exhibit an optimum in the pH range 6.5-7.8, which is little affected by the site and the size of the N-substituent (chapter 6).

A study of the immobilization of aldehyde oxidase to several supports by various methods in order to make continuous operation feasible and to improve the enzyme stability, was undertaken. This showed that of the various matrices and coupling methods tested, the activity of aldehyde oxidase is best retained upon adsorption to modified Sepharose matrices. It is established that the storage stability of enzyme adsorbed to n-alkylamine-substituted Sepharose 4B or diethylaminoethyl Sepharose 6B is significantly lower in comparison to free enzyme. The operational stability of the immobilized enzyme preparation, however, has improved substantially compared to soluble enzyme, although the corresponding productivity is still very poor. The inactivation of aldehyde oxidase during turnover is dependent on the rate of oxidation. Coimmobilization of catalase and/or superoxide dismutase provides a further increase of the operational stability and productivity. A positive effect on both parameters is also found for aldehyde oxidase/n-alkylamine Sepharose 4B preparations by increasing the amount of enzyme adsorbed per unit weight of support (chapter 7). The aldehyde oxidase/Sepharose preparations were used throughout this study to perform small scale syntheses (chapters 2, 3, 5 and 6).

Despite the interesting oxidative capabilities, it is concluded that the application of aldehyde oxidase as an immobilized biocatalyst in organic synthesis is still unattractive at present and awaits further research on the stabilization of this enzyme.

SAMENVATTING

Aldehyde oxydase geïsoleerd uit konijnelever wordt in dit onderzoek bestudeerd met het oog op de toepassing van dit enzym in de organische synthese. Het enzym bezit een brede substraatspecificiteit voor azaheterocyclische verbindingen en biedt zodoende goede vooruitzichten voor een nuttig gebruik.

De oxydatie van *1-alkyl(aryl)-3-aminocarbonylpyridiniumchloriden* door aldehyde oxydase laat zien dat de reactie in principe op twee posities in de pyridiniumring kan plaatsvinden. Van de 1-methyl-, 1-ethyl- en 1-*n*-propylderivaten worden enkel 1-alkyl-1,6-dihydro-6-oxo-3-pyridinecarbonamiden verkregen. De overeenkomstige 4-oxo-verbinding is het enige produkt in de oxydatiereactie met de *t*-butylverbinding, terwijl het *i*-propylderivaat een mengsel van 4- en 6-oxo-produkt oplevert. De kinetiek van de oxydatiereacties levert aanwijzingen voor de aanwezigheid van een hydrofoob gebied in de nabijheid van het aktieve centrum van het enzym (hoofdstuk 2).

Oxydatie van de 1-arylderivaten levert voornamelijk 1-aryl-1,6-dihydro-6-oxo-3-pyridinecarbonamiden op en in geringe mate ook de overeenkomstige 4-oxo-produkten. In het algemeen wordt de oxydatiepositie bepaald door sterische faktoren alhoewel de maximale oxydatiesnelheid van de reaktie met aldehyde oxydase erg gevoelig is voor elektronische effekten. Naarmate de arylsubstituent sterker elektronenzuigend is, neemt de oxydatiesnelheid op C-6 toe. Op deze manier wordt voor vrij aldehyde oxydase een positieve ρ-waarde van ongeveer 3.6 gevonden, wat aangeeft dat een nucleofiele aanval de snelheidsbeperkende stap is in het oxydatiemechanisme van deze aziniumverbindingen. Oxydatie van de 1-arylderivaten door xanthine oxydase uit koeiemelk geeft voornamelijk 4-oxo-produkten. De maximale oxydatiesnelheid wordt voor dit enzym slechts in geringe mate beïnvloed door de aard van de arylsubstituent (hoofdstuk 3).

1-Alkyl(aryl)chinoliniumchloriden worden door aldehyde oxydase eveneens in principe op twee plaatsen geoxydeerd, namelijk op C-2 en C-4. De oxydatiepositie en de maximale oxydatiesnelheid zijn afhankelijk van de grootte en de sterische conformatie van de N-substituent. Echter bij aanwezigheid van een aminocarbonylgroep op C-3 richt de oxydatie zich volledig op de C-4-positie, onafhankelijk van de grootte van de N-substituent (hoofdstuk 5).

Het oxydatiepatroon van verschillende aziniumverbindingen met aldehyde oxydase

wordt vergeleken met de resultaten van covalente aminering van deze verbindingen in vloeibare ammoniak. Hieruit blijkt dat covalente aminering vooral bruikbaar is als een modelreactie voor deze substraten in het geval dat de enzymreactie voornamelijk wordt gecontroleerd door sterische faktoren. Op deze wijze kunnen de oxydatieposities van de 1-alkyl-3-aminocarbonylpyridiniumchloriden vrij goed met deze modelreactie voorspeld worden (hoofdstukken 4 en 5).
N-Methyl- en N-benzylderivaten van *2(1H)- en 4(3H)-pyrimidinonen* (met de substituent op N-1 of N-3) worden door aldehyde oxydase op een overeenkomstige positie geoxydeerd. Onafhankelijk van de grootte van de substituent krijgt men één type produkt van alle geteste substraten, namelijk het overeenkomstige N-1- of N-3-gesubstitueerde uracil. De maximale oxydatiesnelheden voor deze verbindingen geven een optimum te zien in het pH-gebied van 6.5-7.8, dat weinig beïnvloed wordt door de positie en de grootte van de N-substituent (hoofdstuk 6).
Immobilisatie van aldehyde oxydase aan diverse dragermaterialen werd uitgevoerd met behulp van verschillende methoden om een continue procesvoering mogelijk te maken, alsmede om de enzymstabiliteit te verbeteren. Van de bestudeerde dragermaterialen en koppelingsmethoden blijkt adsorptie aan gemodificeerde Sepharosematrices de beste retentie van aldehyde oxydase-aktiviteit te geven. De bewaarstabiliteit van aldehyde oxydase geïmmobiliseerd aan n-alkylamine-gesubstitueerde Sepharose 4B of diethylaminoethyl-Sepharose 6B is aanzienlijk lager dan van vrij enzym. De operationele stabiliteit van deze aldehyde oxydase/Sepharosepreparaten is daarentegen beduidend beter dan van vrij enzym, ofschoon de bijbehorende produktiviteit nog erg laag is. Inaktivering van aldehyde oxydase tijdens de katalyse is afhankelijk van de oxydatiesnelheid. Coïmmobilisatie van katalase en/of superoxyde dismutase draagt bij tot een verbetering van de operationele stabiliteit en produktiviteit. Een hogere belading van het dragermateriaal met enzym heeft eveneens een gunstige invloed op deze beide faktoren (hoofdstuk 7). De aldehyde oxydase/Sepharose-preparaten werden in dit onderzoek gebruikt voor het uitvoeren van synthesen op kleine schaal (hoofdstukken 2, 3, 5 en 6).
Ondanks de interessante oxydatiemogelijkheden met aldehyde oxydase, is op het moment de toepassing van dit enzym als geïmmobiliseerde biokatalysator in de preparatieve organische chemie nog onaantrekkelijk. Verder onderzoek met betrekking tot de stabilisatie van het enzym is daarom geboden.

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