The Oxidation of 1-Alkyl(aryl)quinolinium Chlorides with Rabbit Liver Aldehyde Oxidase [1]

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1-Alkyl(aryl)quinolinium chlorides are oxidized by rabbit liver aldehyde oxidase at 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*-1 substituent. The presence of a 3-carboxamido group directs the oxidation completely to position C-4, irrespective of the size of the *N*-substituent. Application of covalent amination in liquid ammonia as an "enzyme model" for the oxidation of these compounds shows little resemblance.

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The oxidation of azaheterocyclic compounds with the molybdenum iron-sulphur containing flavoproteins xanthine oxidase [2] from bovine milk and aldehyde oxidase [2] from rabbit liver for synthetic purposes is a current subject of investigation in our laboratories [1,3,4]. Rabbit liver aldehyde oxidase has been successfully used to oxidize a number of 1-alkyl(aryl)-3-aminocarbonylpyridinium chlorides [1,3] 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 [1].

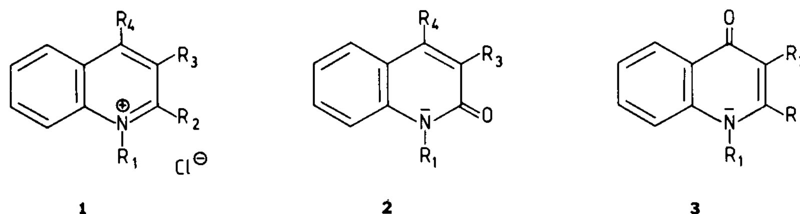
As an extension of this work we report in this paper on the oxidation of 1-alkyl(aryl)quinolinium compounds (Scheme 1) by rabbit liver aldehyde oxidase. These compounds can be regarded as extended pyridinium analogs and therefore very probably will be oxidized in the same manner with regard to the influence of the N-1 substituent.

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].

Enzymic Oxidation of 1-Alkyl(aryl)quinolinium Chlorides **1a-g**.

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-quinolone (**2a**); only a small amount of 1-methyl-4-quinolone (**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 quinolones **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 oxida-

Scheme 1



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

tion at the different sites of the compounds **1a-c** were measured at *p*H 9.0 and the results are collected in Table 1. 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

Table 1

Maximum Rates for the Oxidation of 1-Alkyl(aryl)quinolinium Chlorides **1a-g** by Free Aldehyde Oxidase at *p*H 9.0 [a]

Substrate	V 2-oxo [b]	V 4-oxo [b]
1a	0.66 ± 0.03	[c]
1b	0.043 ± 0.005	0.34 ± 0.03
1c		0.63 ± 0.04
1d		[c]
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 [3] with this aldehyde oxidase preparation was 0.42 ± 0.02 μmole /min mg [b] In μmole /minute mg [c] No accurate data could be determined for oxidation at this site.

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 [1].

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 $\triangle\delta(H_2\text{-}H_4)$ values. These $\triangle\delta$ values reflect the steric influence on 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 $\triangle\delta(H_2\text{-}H_4)$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 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, expecially for R = Cl, conformation II will be more favorable than I, leading to the shielding effect of the H-2 of the quinolinium ring by the 2,6-dichlorobenzyl group. Similar results were acquired before for 3-aminocarbonyl-1-(2,4,6-trimeth-

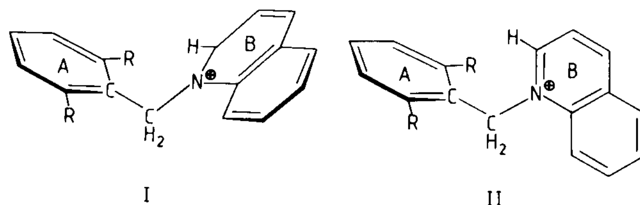


Figure 1. Two conformations of compounds **1b** and **1c** resulting from rotation about the C-N bond.

ylbenzyl)pyridinium chloride [1]. We suggest that the orientation of the 2,6-dichlorobenzyl group as indicated in Figure 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 analogs 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 [1], 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 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-quinolone (**3f**). If however the 1,4-dimethylquinolinium compound **1g** is oxidized, the oxidation only takes place at C-2. The maximum oxidation rates of both quinolinium chlorides **1f** and **1g** are lower than those of the parent compound **1a** (Table 1). The low rate for the 1,2-dimethylquinolinium chloride **1f** is of course due to the fact that the favored 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

Table 2

^{1}H-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° [a]

Compound	H-2	△δ[b]	H-3	△δ[b]	H-4	△δ[b]	N-CH$_3$	△δ[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]	—	.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 deuterium oxide. [c] C-2(CH$_3$): 1.69 ppm (△δ = 1.45). [d] C-4(CH$_3$): 1.96 ppm (△δ = 1.05). [e] After incubation at room temperature. [f] Not interpretable due to overlap by the proton signals of the benzo ring.

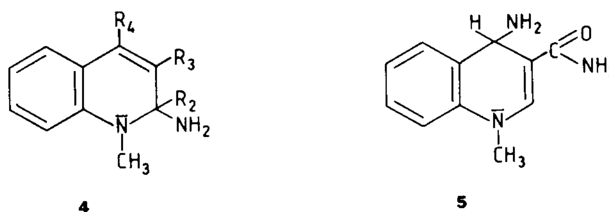
compound **1a**, as we have already mentioned. This effect is also operative in the oxidation of 1,4-dimethylquinolinium chloride which shows a maximum rate of about 3.7 times lower than the rate for 1-methylquinolinium chloride. In the nucleophilic substitution reaction of 2-chloroquinoline with methoxide ions a decrease in reaction rate of a factor of 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 *p*H range 6.4-9.7. The rate gradually increases from *p*H 6.4 to 7.7 and is about constant over the *p*H range 7.7-9.7. This *p*H profile is quite similar to that found for 3-aminocarbonyl-1-phenylpyridinium chloride [1].

With immobilized aldehyde oxidase [3] oxidation of the quinolinium salts **1a-c** to the respective quinolones was performed on a small preparative scale [12]. Total product yields varying from 34 to 48% have been obtained (Table 4); these yields are rather low compared to those acquired in the conversions of 3-aminocarbonyl-1-arylpyridinium chlorides [1].

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 intermediate during the catalytic reaction of aldehyde oxidase [1]. 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 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 3), which were compared with corresponding data of 1-alkyl(aryl)-3-aminocarbonylpyridinium chlorides and their σ-aducts [5].

Table 2 clearly shows that the covalent amination of compounds **1a**, **1f** and **1g** takes place only at C-2 [13], resulting in the corresponding adducts **4** (Scheme 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 2). At room temperature adducts **4a, 4f** and 4g are unstable; most likely they undergo ring-opening reactions [5]. The results indicate



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 2

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°. 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° gives the C-2 adduct **4d**. At higher temperatures (>-40°) 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 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 favored adduct which

Table 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
	$\Delta\delta$	83.8	17.6	10.7
5 [b]	NH_3	138.9	46.3	38.7
	$\Delta\delta$	10.9	99.0	7.2

[a] At -50°. [b] After incubation at room temperature.

at higher temperature partly converts into its isomer **5**. 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 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.

EXPERIMENTAL

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. The ^{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 18μs, respectively. All nmr data were converted to the DSS/TMS scale.

The hplc analysis was performed with a Varian 5000 instrument equipped with a Micro Pak MCH-10 column (30 × 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/minute.

The 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 [3]. 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 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 m*M* potassium phosphate buffer, *p*H 7.8 containing 0.1 m*M* EDTA. Only fractions free of aldehyde oxidase activity were pooled.

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-quinolium (R = methyl [17], benzyl [18], 2,6-dichlorobenzyl [19]), 1,2-dimethylquinolinium [20], 1,4-dimethylquinolinium [21], 3-aminocarbonyl-1-R-quinolinium (R = methyl, benzyl [22]) bromide or iodide, 3-aminocarbonyl-1-methylpyridinium chloride [3], 3-aminocarbonyl-4-deuterio-1-methylpyridinium chloride (74% deuteration according to nmr analysis) [23], 1-methyl-2-quinolone [24], 1,4-dimethyl-2-quinolone [25], 1-methyl-4-quinolone [26] and 1,2-dimethyl-4-quinolone [27] were synthesized according to known synthetic procedures. 4-Quinolone trihydrate was purchased from Aldrich.

1-Benzyl-4-quinolone (**3b**) and 1-(2,6-Dichlorobenzyl)-4-quinolone (**3c**).

4-Quinolone 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 in the case of **3b** or purified by column chromatography in the case of **3c** with dichloromethane-ethylacetate (1:1) as eluent followed by crystallization from ethanol. In this manner **3b** was synthesized (48%), mp 123-124° (lit [28] 124-125°). Compound **3c** was synthesized by this method (60%), mp 198.5-199.5°.

Anal. Calcd. for $C_{16}H_{11}Cl_2NO$: C, 63.18; H, 3.64. Found: C, 62.88; H. 3.65.

1-Methyl-4-oxo-1,4-dihydroquinoline-3-carboxamide (**3d**) and 1-Benzyl-4-oxo-1,4-dihydroquinoline-3-carboxamide (**3e**).

1-R-3-quinolinecarboxylic acid-4-one [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 ethylchloroformate was added dropwise with stirring at -5°. After one hour 30 ml of 33% ammonia was added, the precipitate filtered off (yield 80%) and recrystallized twice from ethanol. In this manner compound **3d** was prepared; mp 286-288°.

Anal. Calcd. for $C_{11}H_{10}N_2O_2$: C, 65.33; H, 4.98. Found: C, 65.22; H, 4.82.

Compound **3e** was also prepared, mp 232-233°.

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 [3]. 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 Quinolones **2a**, **2b**, **3a-c** by Immobilized Aldehyde Oxidase.

Rabbit liver aldehyde oxidase was immobilized by adsorption 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 m*M* potassium phosphate buffer, *p*H 7.8 (0.1 m*M* EDTA) at 4°. 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 m*M* substrate solution in the same buffer (32 mg of substrate applied) was slowly (0.25 ml/min) recirculated through the column at 4° 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 *p*H 6.5, evaporated to dryness and the residue purified by column chromatography (eluents dichloromethane and ethylacetate). Uv and mass spectra of **2a** and **3a-c** were identical to those of authentic materials. For compound **2b** no authentic sample was available; however, 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 4.

Table 4

Product Yields and Exact Mass Data of Some 1-Alkyl(aryl)quinolones Obtained by Oxidation Using Immobilized Aldehyde Oxidase

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

[a] After recrystallization from ethanol mp 50-51° (lit [31] 50-510).

Kinetic Assays.

The assay for aldehyde oxidase was carried out as described previously [3]. Each assay was at least performed in duplicate. As buffer sodium borate, *p*H 9.0 with an ionic strength I = 0.05, including 0.1 m*M* EDTA, was used. For assays in the *p*H 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△ε) 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 *p*H 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 μmole of 3-aminocarbonyl-1-methylpyridinium chloride per minute at 25°. The assay conditions were: 5 m*M* substrate in 50 m*M* potassium phosphate buffer, *p*H 7.8 (0.1 m*M* EDTA) with the reaction being monitored at 292 nm (log △ε = 3.64). Kinetic data were calculated from Lineweaver-Burk plots [33].

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