# The Oxidation of 6- and 7-Aryl-4(3*H*)-pteridinones by Immobilized Arthrobacter M-4 Cells Containing Xanthine Oxidase [1]



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The preparation of 6- and 7-(*p*X-phenyl)-4(3*H*)-pteridinones (X = H, $CH_3$, $OCH_3$) is described. The oxidation of these compounds by (immobilized) Arthrobacter M-4 cells containing xanthine oxidase has been studied. The oxidation monitored by uv spectroscopy usually goes fast, except for 7-(*p*X-phenyl)-4(3*H*)-pteridinones (X = $CH_3$, $OCH_3$), which are slowly oxidized. With bacterial cells immobilized in gelatine crosslinked with glutaraldehyde small laboratory-scale oxidations were carried out. Based on spectral data the products of the oxidation reactions are 6- and 7-aryllumazines.

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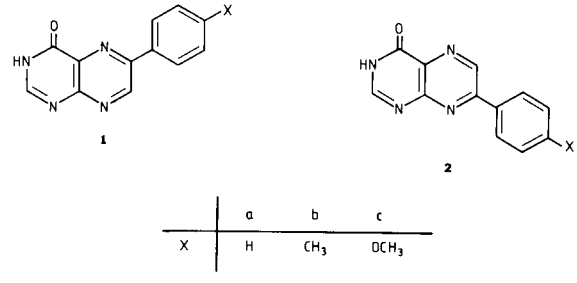
Since many years there are ongoing studies in the laboratory of Organic Chemistry at Wageningen on the potential application of enzymes for functionalisation of azaheteroaromatics [2-9]. Enzymes under study are xanthine oxidase [3], xanthine dehydrogenase [4], aldehyde oxidase [1,2,5] and more recently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

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

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

Scheme 1



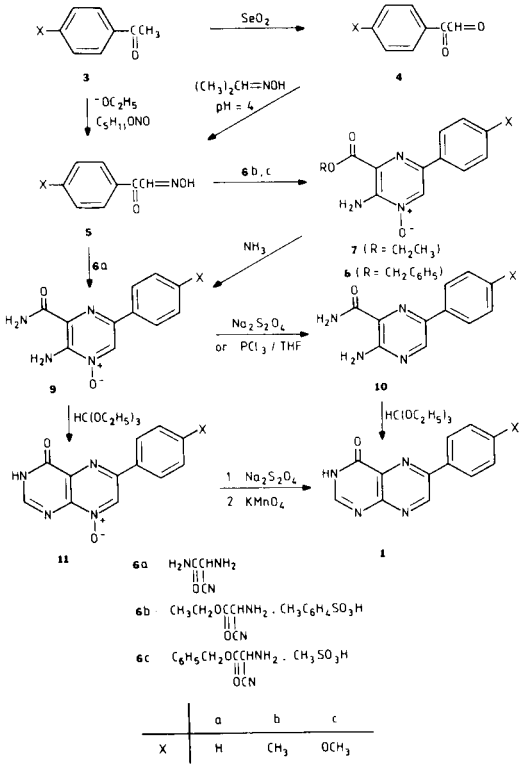

Synthesis.

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

Condensation of 4,5-diamino-6(1*H*)-pyrimidinone with phenylglyoxal at *p*H = 7.5 gives almost exclusively the 7-phenyl compound **2a**; by recrystallization from dimethylsulfoxide pure **2a** could be obtained. In a similar way the 7-aryl compounds **2b** and **2c** are prepared. Performing the condensation at *p*H = 2.7 results in a mixture of **1a** (70%)

and **2a** (30%) [13]. The separation of **1a** from **2a** is, however, difficult, time-consuming and lowers the yield considerably. Since in our experiments we wanted to dispose of 6-aryl-4(3*H*)-pteridinones **1** not contaminated with the 7-aryl-4(3*H*)-pteridinones **2** we turned to the versatile synthetic route [17] designed by Taylor and co-workers, which fully served our purpose. The reaction scheme is outlined in Scheme 2.

Scheme 2

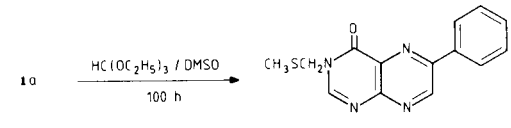



The synthesis required arylglyoxal-2-oximes **5** which were obtained by a selenium dioxide oxidation of the para substituted acetophenones **3** [18] and subsequent transoximation of the arylglyoxals **4** with acetonoxime. The best results were obtained when compounds **4** are immediately used after preparation; if necessary to keep them some time, they were converted into their hydrates [19]. Direct preparation of **5** from **3** by a base-catalyzed reaction with sodium ethoxide and pentylnitrite [21] proved in our hands to be tedious and unpredictable: the yields were consistently poor, the reaction time was depending on the para substituent and variable amounts of the corresponding benzoic acid [21b] were formed. Oximation of **4** with hydroxylamine instead of acetonoxime usually resulted in the formation of the dioxime of the arylglyoxals as main product [22]. Acetoxime was found to be efficient and highly selective in producing the desired compounds **5** in good, reproducible yields [24].

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

Two methods were applied to convert **9** into the desired 6-aryl-4(3*H*)-pteridinones **1** [17] as indicated in Scheme 2. Reaction of **9** with triethyl orthoformate in dimethylformamide or dimethylaceetamide yielded the 6-aryl-4(3*H*)-pteridinone 8-oxides **11**. The yields in dimethylformamide were found to be higher than in dimethylaceetamide. Treatment of **11** with sodium dithionite and subsequent mild oxidation with potassium permanganate gave the expected pteridinones **1**. Oxidation by potassium permanganate after dithionite treatment is necessary, since dithionite not only performs deoxygenation, but also reduction of the pyrazine ring, as indicated by mass spectrometric data. Alternatively, **9** could first be deoxygenated by either sodium dithionite or phosphorus trichloride into the 2-amino-3-carbamoyl-5-(*p*X-phenyl)pyrazines **10** followed by heating with triethyl orthoformate in acetic anhydride at 150° to achieve cyclization into **1**. The reduction of **9** into **10** by treatment with boiling water containing sodium dithionite gives higher yields than the reaction with phosphorus trichloride, although the former method requires 24 hours of reaction time while only two hours are needed in the latter case. It has been found that, when the cyclization of **10a** into **1a** was not performed in acetic anhydride but in dimethylsulfoxide under strenuous conditions, besides cyclization a methylthiomethyl group is introduced at N-3: reacting **10a** with triethyl orthoformate at 110° in dimethylsulfoxide for 16 hours gave only partial cyclization into **1a**, but by prolonging the reaction time from 16 to 100 hours 3-methylthiomethyl-6-phenyl-4(3*H*)-pteridinone (**12**) was formed in 64% yield. Structure **12** was assigned by nmr spectroscopy and microanalysis. The meth-

Scheme 3

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ylthiomethylation most probably proceeds after ring-closure into **1a**; the reaction involves the methylenesulfonium cation [28].

In the $^1$H-nmr spectra of the pure 6- and 7-aryl-4(3*H*)-pteridinones the chemical shift of the proton at C-7 in **1** was found to be about 0.2 ppm more upfield than the proton at C-6 in **2** [13]. The difference in the $^{13}$C-chemical shift of C-6 and C-7 in **1** is about 3 ppm while interestingly the difference in chemical shift of C-6 and C-7 in the 7-arylderivative **2** is about 13 to 14 ppm. In Table 1 the chemical shifts of the 6- and 7-arylderivatives are compared with those of 4(3*H*)-pteridinone (**15**) indicating the effect of the aryl group on the resonance of both C-6 and C-7. Similar differences were also observed before [13] confirming the structures of the compounds **1** and **2**. In the pteridine 8-oxides **11** the chemical shift of H-7 is shifted about 0.25 ppm upfield relative to H-7 in **1**, but remarkably in the pyrazine 1-oxides **9** the chemical shift of H-6 is moved 0.25 ppm downfield in comparison with H-6 in the pyrazines **10**. Mass spectra of the compounds **1** and **2** also revealed disparities. In the spectra of **1a-c** a peak for $(M-27)^+$ is found, while in the spectra of **2a-c** always a peak for $M^+ = 121$ is present. The spectra of both the 6- and 7-aryl-derivatives show a peak corresponding with $(M-120)^+$.

Table 1

$^{13}$C NMR Data of 6- and 7-Aryl-4(3*H*)-pteridinones **1a-c** and **2a-c**

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

Introduction of an electron-donating substituent at the para-position of the phenylring resulted in a bathochromic shift in the uv-spectra of the pteridines. A bathochromic shift of about 25 nm resulted from the introduction of the *N*-oxide in the pteridine nucleus (Table 2).

Enzymatic Oxidation.

The production and characterization of Arthrobacter X-4 cells has already been published [8]. In a similar way Arthrobacter M-4 cells were grown [9]. Before using the cells, they were disrupted by ultrasonification and after

Table 2

Ultraviolet Spectra for 6-, 7-Aryl-4(3*H*)-pteridinones and 6-Aryl-4(3*H*)-pteridinone 8-Oxides [λ (log ε)] [a]

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

[a] Each compound was dissolved in the minimal amount of 0.01 *M* potassium hydroxide and from this stock solution (40 mg/100 ml) the final dilution was made in the appropriate buffer. [b] Sodium acetate buffer. [c] Tris-HCl buffer. [d] Potassium phosphate buffer. [e] sh = shoulder.

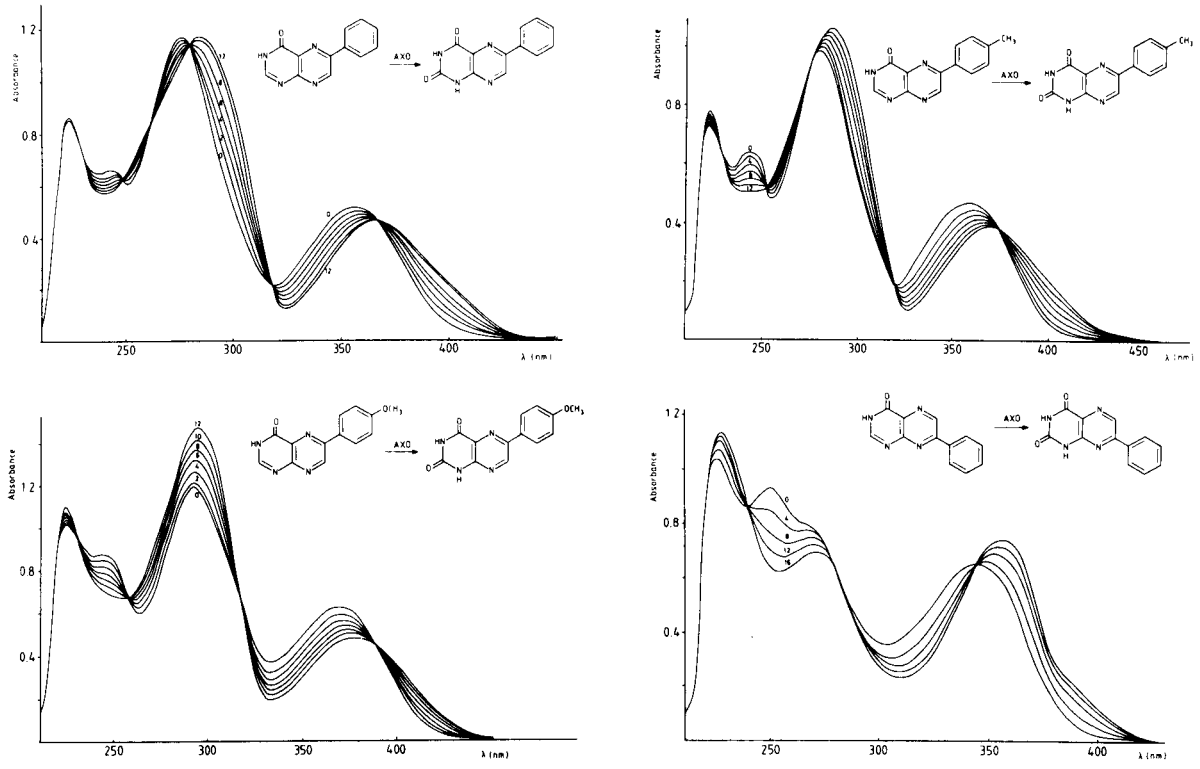
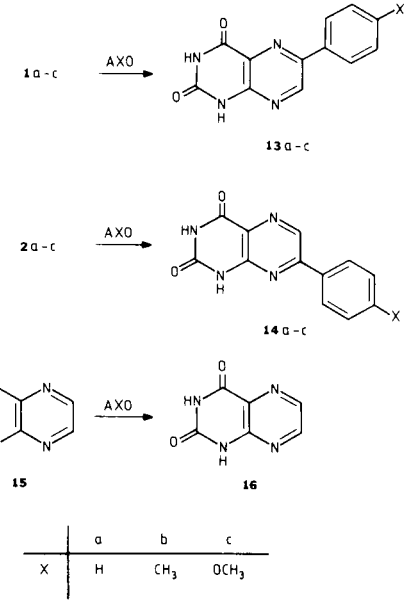


Figure 1. Ultraviolet spectra, as recorded for $5.10^{-5}$ *M* solutions of **1a**, **1b**, **1c** and **2a** after the addition of 190 $\mu$g of AXO. Spectra were periodically scanned during the reaction (the time in minutes is indicated by the numbers above the lines).

centrifugation the supernatant contained the crude AXO enzyme extract. Incubation of 6-phenyl-4(3*H*)-pteridinone (**1a**) with this extract and following the reaction in time by scanning the uv-spectrum of the solution at regular intervals, resulted in a spectrum with sharp isosbestic points (Figure 1). When the uv-spectrum of the reaction mixture did not change anymore, the uv-spectrum obtained was identical to that of 6-phenyllumazine (**13a**), as shown by comparison with a specimen prepared independently [30]. Further incubation of the solution containing **13a** with an additional aliquot of bacterial xanthine oxidase gave no further changes in the uv-spectrum indicating that **13a** is stable towards further oxidation. These results indicate that in the enzymatic oxidation of **1a** only one product and no by-products are formed. This result is not unexpected since the carbon atom between two ring nitrogens in pteridines and purines is highly susceptible [11,31,32] to enzymatic oxidation and the unoccupied C-7 position in **1a** is sterically hindered by the phenyl-group at C-6. Incubation of the other 6-arylpteridinones **1b** and **1c** with the enzyme extract showed that also these 6-aryl derivatives were easily oxidized into the corresponding lumazines **13b** and **13c**. From the 7-arylpteridinones **2a-c** only **2a** was oxidized at a measurable rate. After incubation overnight at room temperature only **2b** did show a slow change in the UV-spectrum.

Scheme 4




Since the enzymatic oxidation resulted in the formation of only one product and not in a mixture of products we in-

Table 3

$^{13}$C NMR Data of 6- and 7-Aryl-2,4(1*H*,3*H*)-pteridinediones **13a-c** and **14a-c**

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

[a] These signals may be interchanged.

vestigated whether these lumazines could be prepared on a small laboratory scale (50 to 70 mg) using immobilized Arthrobacter M-4 cells. For this purpose the cells were immobilized in gelatine crosslinked with glutaraldehyde by the method previously described [7]. In order to enhance the solubility of the substrates **1a-c**, the enzymatic oxidation of these compounds with immobilized Arthrobacter cells was carried out at *p*H = 8.0, although the optimal *p*H for this bacterial enzyme is around 7.2. Oxidation for about one week at room temperature resulted in the complete conversion of **1** into **13**. The structures of the products were identified by comparison of the nmr data, uv-data and mass specrometric data with those of an authentic specimen (Tables 3 and 4).

From the 7-arylpteridinones **2a-c** only **2a** was rather quickly converted, in contrast to its *p*-methylphenyl and *p*-methoxyphenyl derivatives **2b** and **2c**. However, after five days of incubation with immobilized cells, **2b** gave 70% of oxidized product, while from **2c** after three weeks of incubation at room temperature with immobilized cells only 15% of the corresponding lumazine **14c** was formed, as deduced from $^1$H nmr spectroscopy.

The enzymatic conversions of **1a-c** and **2a** were all quantitative but some losses occurred during the isolation procedure. The yields of the isolated products varied from 86 to 96%. Table 4 summarizes the yields and analytical data of the lumazines obtained in the reactions with immobilized cells.

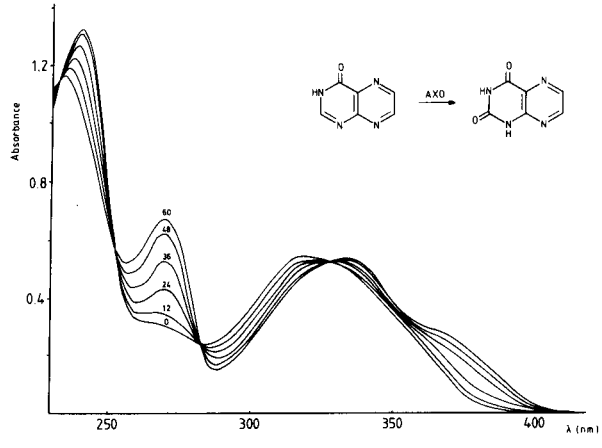



Figure 2. Ultraviolet spectra, as recorded for $8.10^{-5}$ *M solution of 4(3H)*-pteridinone (**15**) after the addition of 190 *μg* of AXO. The spectra were taken every twelve minutes. After one hour the reaction was complete.

Table 4

Yields and analytical data of products obtained by oxidation using immobilized Arthrobacter M-4 cells

| Substrate | Product | Yield | $^1$H NMR Data [δ DMSO-d$_6$] | Ultraviolet Spectra [λ (log ε)] | | | | Formula | Exact mass Exp. | Exact mass Theor. |
|---|---|---|---|---|---|---|---|---|---|---|
| **1a** | **13a** | 92% | 7.50-7.80 (m, 3H, ArH), 8.05-8.25 (m, 2, ArH), 9.23 (s, 1H, H-7), 11.70 (br s, 1H, NH), 11.92 (br s, 1H, NH) | *p*H = 5 [a] | | 273 (4.36) | 354 (4.01) | | | |
| | | | | 8 [b] | | 286 (4.30) | 364 (3.92 | $C_{12}H_8N_4O_2$ | 240,0648 | 240,0647 |
| | | | | 13 [c] | | 280 (4.39) | 384 (3.98) | | | |
| **1b** | **13b** | 87% | 2.43 (s, 3H, $CH_3$), 7.66 (d, J = 7.5 Hz, 2H, ArH), 8.05 (d, J = 7.5 Hz, 2H, ArH), 9.27 (s, 1H, H-7), 11.80 (br s, 2H, NH) | 5 | | 279 (4.40) | 361 (4.00) | | | |
| | | | | 8 | | 289 (4.36) | 369 (3.93) | $C_{13}H_{10}N_4O_2$ | 254,0806 | 254,0804 |
| | | | | 13 | | 286 (4.38) | 388 (3.98) | | | |
| **1c** | **13c** | 86% | 3.87 (s, 3H, $OCH_3$), 7.10 (d, J = 9 Hz, 2H, ArH), 8.08 (d, J = 9 Hz, 2H, ArH), 9.20 (s, 1H, H-7), 11.65 (br s, 1H, NH), 11.90 (br s, 1H, NH) | 5 | | 288 (4.40) | 376 (3.93) | | | |
| | | | | 8 | | 294 (4.42) | 375 (3.92) | $C_{13}H_{10}N_4O_3$ | 270,0754 | 270,0753 |
| | | | | 13 | | 298 (4.46) | 384 (3.96) | | | |
| **2a** | **14a** | 96% | 7.50-7.73 (m, 3H, ArH), 8.10-8.36 (m, 2H, ArH), 9.17 (s, 1H, H-6), 11.63 (s, 1H, NH), 11.95 (br s, 1H, NH) | 5 | 223 (4.33) | 260 (3.98) sh [d] | 349 (4.33) | | | |
| | | | | 8 | 226 (4.33) | 271 (4.11) | 353 (4.19) | $C_{12}H_8N_4O_2$ | 240,0649 | 240,0647 |
| | | | | 13 | 230 (4.35) | 268 (4.32) | 372 (4.07) | | | |
| **2b** | **14b** | 70% [e] | 2.48 (s, 3H, $CH_3$), 7.43 (d, J = 7.5 Hz, 2H, ArH), 8.15 (d, J = 7.5 Hz, 2H, ArH), 9.12 (s, 1H, H-6), 11.65 (br s, 1H, NH), 11.92 (br s, 1H, NH) | 5 | 230 (4.29) | 256 (3.89) sh | 280 (3.81) sh | 355 (4.38) | | |
| | | | | 8 | 231 (4.23) | 274 (4.02) | | 358 (4.26) | $C_{13}H_{10}N_4O_2$ | 254,0801 | 254,0804 |
| | | | | 13 | 236 (4.32) | 270 (4.24) | 300 (3.93) sh | 372 (4.12) | | |
| **2c** | **14c** | 15% [e] | 3.92 (s, 3H, $OCH_3$), 7.29 (d, J = 9 Hz, 2H, ArH), 8.33 (d, J = 9 Hz, ArH), 9.15 (s, 1H, H-6), 11.63 (br s, 1H, NH), 11.88 (br s, 1H, NH) | 5 | 235 (4.22) | 268 (3.80) sh | 300 (3.73) | 367 (4.39) | | |
| | | | | 8 | 235 (4.23) | 272 (3.94) sh | 304 (3.81) sh | 369 (4.30) | $C_{13}H_{10}N_4O_3$ | 270,0755 | 270,0753 |
| | | | | 13 | 244 (4.27) | 270 (4.12) sh | 313 (3.90) sh | 378 (4.17) | | |

[a] Sodium acetate buffer. [b] Tris-HCl buffer. [c] 0.1 M potassium hydroxide. [d] sh = shoulder. [e] Deduced from 300 MHz $^1$H nmr specrum taken after isolation from the crude reaction mixture obtained after separation of the immobilized cells.

The kinetic constants Vm and Km were determined for the AXO-mediated reaction of **1a** and **2a** at *p*H = 8.0 assuming that the reaction follows simple Michaelis-Menten kinetics. For reasons of comparison we also determined the kinetic parameters of 4(3*H*)-pteridinone (**15**). This compound is exclusively oxidized at the C-2 position, since the uv-spectrum after the oxidation of **15** is identical to that of lumazine (**16**). Lumazine is not further oxidized by the bacterial xanthine oxidase. This is in contrast to the behaviour of **15** towards bovine milk xanthine oxidase, which resulted in the formation of 2,4,7(1*H*,3*H*,8*H*)-pteridinetrione *via* 4,7(3*H*,8*H*)-pteridinedione [31b]. For this reason the comparison between **1a**, **2a** and **15** is completely justified, since oxidation takes place at the same carbon atom. For the 6-phenyl derivative **1a** the maximum rate of oxidation at C-2 is about two times higher than that at C-2 in the 7-phenylpteridinone **2a**. The Michaelis constant Km of the compounds **1a** and **2a** are about ten times lower than the corresponding values found for 4(3*H*)-pteridinone and xanthine [33]. The position of the phenyl group in the pteridine is apparently important for adequate fitting of the substrate in the active site of the enzyme. In Table 5 also the Vm/Km values are listed. These figures reflect the relative efficiency of enzymatic oxidation for each of these substrates. Even then, the increase in relative efficiency is largely due to the presence of the phenyl group in both **1a** and **2a** and point to the existence of a hydrophobic regio in the vicinity of the active center of the bacterial enzyme; this proposal parallels that of Baker *et al.* [34] for bovine milk xanthine oxidase.

Table 5

Kinetic Parameters for the Oxidation of **1a**, **2a** and **15** by the Crude Cell-Free Extract at *p*H = 8.0 [a]

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

[a] The oxidation rate for 1-methylxanthine with the bacterial xanthine oxidase preparations used here was 0.25 μmol ± 0.03 μmol/minute mg. [b] In μmol/ℓ. [c] In μmol/minute mg. [d] In ml/minute mg.

Table 6

Yields of 2-Amino-3-aminocarbonyl-5-arylpyrazine Oxides **9a-c** from the Compounds **5**, **7**, **8** by the methods A, B and C

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

## EXPERIMENTAL

Melting points were determined on a Kofler hot stage equipped with a microscope and a polarizer and they are uncorrected. The $^1$H nmr spectra were measured using a Hitachi Perkin-Elmer R-248 or a Varian EM 390 spectrometer, with TMS as internal standard. The $^{13}$C nmr spectra were recorded using dimethylsulfoxide-$d_6$ as solvent and internal standard on a Bruker CXP 300 spectrometer equipped with a B-VT 1000 variable temperature controller. Infrared spectra were obtained with a Perkin Elmer 237 or a Hitachi EPI-G3. Mass spectra were recorded on an AEI MS 920 instrument. Field desorption mass spectra were taken of all the compounds with an *N*-oxide and a mp. higher than 200°, most of the time the molecular ion was observed with a small intensity for (M-16)$^+$, unlike the spectra taken with the electron impact technique, where deoxygenation of these compounds occurred. Ultraviolet spectra were determined using a Beckman DU-7, Aminco DW-2A or Varian DMS 100 spectrophotometer. Column chromatography was carried out over Merck Silica gel 60 (70-230 mesh ASTM) and Silical GF from Merck was used for analytical thin layer chromatography, using the following solvent systems: A, chloroform-ethanol (95:5); B, chloroform-methanol (9:1); C, dichloromethane-methane (9:1); D, benzene-ethyl acetate (7:3).

### Preparation of Starting Materials and Reference Compounds.

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

### Ethyl 2-Amino-2-cyanoacetate *p*-Toluenesulfonic Acid Salt (**6b**) [37,38].

This compound was synthesized using the modification [25,26] of previously described methods which were adapted for preparation on a large scale. Under nitrogen ethyl 2-oximino-2-cyanoacetate [39] (60 g, 0.42 mole, mp 133°) was covered with 360 ml of water. The suspension was carefully treated with 180 ml of a saturated sodium bicarbonate solution. To the yellow solution sodium dithionite (90%, 227 g, 1.17 mmoles) was added in portions over a period of about an hour. The temperature rose about 20° during this time, but was kept below 35° to avoid lower yields or impure product. After the addition, stirring was continued for 30 minutes. Extraction with five portions of distilled chloroform (5 × 300 ml), drying of the extracts over magnesium sulfate and evaporation *in vacuo* (below 35°) yielded 32-36 g of an oil (59-66%). This oil was immediately diluted with 300 ml of dry ether. To this solution *p*-toluenesulfonic acid monohydrate (60 g, 0.32 mole) in 150 ml of ethanol was added. Under vigorous stirring the solution was slowly diluted with dry ether to 1000 ml to induce crystallization. The mixture was kept overnight at −20°. After filtering, washing with cold dry ether and drying over phosphorus pentoxide, 65-70° g of a snow-white precipitate was obtained (48-52%), mp 118-120° (lit [38] mp 125-126°, lit [37] mp 115-117°); it was used without further purification in the cyclisation reactions.

### Arylglyoxal 2-Oximes **5a-c**.

Selenium dioxide (37 g, 0.33 mole) was mixed with 10 ml of water and 250 ml of dioxane at 50-60°. When almost all selenium dioxide had gone into solution 0.3 mole of **3** was added in one portion. After refluxing for 16 hours the solution was filtered hot to remove elemental selenium and the yellow-orange filtrate was concentrated *in vacuo*. The resulting reddish yellow oil was diluted with 300 ml of chloroform and washed quickly with a saturated sodium bicarbonate solution (100 ml) and water (100 ml). After drying over magnesium sulfate, any precipitate formed during this time was removed by filtration through Celite. The yellow coloured solution turned reddish-yellow due to formation of spots of amorphous red selenium. After evaporation of the chloroform, the resulting oil was purified by distillation *in vacuo* (**4a** 70-72%, bp 90-95° at 20 mm Hg, lit [20b] bp 95-97° at 25 mm Hg; **4b**: 81-83%, bp 76-80° at 3 mm Hg, lit [40] bp

104-110° at 10 mm Hg; **4c**: 69-72%, bp 145-147° at 13 mm Hg, lit [40] bp 105-110° at 3 mm Hg). The resulting yellow oil (for **4c**: the oil solidified giving a product with mp 100-104°) was converted into the hydrate by refluxing in 400 ml of water (800 ml for **4c**) during 3 hours. Charcoal was added and after keeping at reflux for ten minutes the solution was filtered. After standing overnight at 4° the white precipitate was isolated by filtration. After washing with ice-water the hydrate [41] was dried over phosphorus pentoxide (hydrate of **4a**; 80%, mp 70-74°, lit [20b] mp 71°, lit [42] mp 76-77°; hydrate of **4b**: 75%, mp 90-94°, then mp 105-108°, lit [42] mp 98-99°; hydrate of **4c**: 72%, mp 75-80°, then mp 110-114°, lit [42] 126-128°, lit [43a] 89-92°, lit [44] mp 84°). When the product is not converted into the hydrate, di- and polymerization is likely to occur, even when the arylglyoxal is kept at −30° (as indicated by nmr spectroscopy). For conversion into **5a-c** also freshly distilled **4a-c** can be used.

Method A.

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

Method B.

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

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

Phenylglyoxal 2-Oxime (Phenylglyoxalaldoxime) (**5a**).

This compound was obtained in a yield of 82% (method A), 91% (method B), mp 127-128° (mp [45] 126-127°).

(*p*-Methylphenyl)glyoxal 2-Oxime (**5b**).

This compound was obtained in a yield of 80% (method A), 93% (method B), mp 98-100° (lit [46] 100°).

(*p*-Methoxyphenyl)glyoxal 2-Oxime (**5c**).

This compound was obtained in a yield of 86% (method A), 92% (method B), mp 117-118° (lit [21b] mp 119°).

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

In 10 ml of anhydrous methanol a mixture of compound **5b** (1.7 g, 10.4 mmoles) and **6b** (3.2 g, 10 mmoles) was stirred at 35° for five days. After cooling in ice-water for thirty minutes the yellow precipitate was filtered, washed with ice-cold methanol (10 ml) and cold ether (2 × 30 ml) to give 1.83 g (67%) of a bright yellow solid. Work-up after ten days of stirring gave a yield of 83%. Concentrating of the filtrate and extraction with ethyl acetate and chloroform [17] yielded an additional amount of 0.15-0.20 g of **7b**. Recrystallization from 2-propanol gave bright yellow needles of **7b** with mp 173-175°; $^1$H nmr (90 MHz, deuteriochloroform): δ 1.48 (t, J = 7.1 Hz, 3H, $CH_3$), 2.41 (s, 3H, $CH_3$), 4.48 (q, J = 7.1 Hz, 2 H, $CH_2$), 7.33 (d, J = 7.5 Hz, 2H, ArH), 7.35 (br s, 2H, $NH_2$), 7.76 (d, J = 7.5 Hz, 2H, ArH), 8.63 (s, 1H, H-6).

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

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

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

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

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

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

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

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

This compound was prepared according to the method described for **8a**, starting from **5b** (1.80 g, 10.9 mmoles) and **6c** (2.86 g, 10 mmoles) in 15 ml of anhydrous methanol. Stirring for five days produced 2.75 g (81%) of a golden yellow solid. Filtration and recrystallization from 1-propanol afforded bright yellow, fluffy needles of **8b**, mp 184-186°; $^1$H nmr (deuteriochloroform + DMSO-$d_6$): δ 2.37 (s, 3H, $CH_3$), 5.47 (s, 2H, $CH_2$), 7.23 (d, J = 7.5 Hz, 2H, ArH), 7.35-7.56 (m, 5H, ArH), 7.65 (br s, 2H, $NH_2$, deuterium oxide exchangeable), 7.83 (d, J = 7.5 Hz, 2H, ArH), 8.90 (s, 1H, H-6).

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

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

This compound was synthesized according to the same procedure as given for **8a**, using **5c** (2.01 g, 11.2 mmoles) and **6c** (2.86 g, 10 mmoles) in 15 ml of anhydrous methanol. A bright yellow solid (2.58 g, 76%) was obtained after five days of stirring. Filtration and recrystallization from 1-propanol yielded bright yellow, fluffy needles of **8c**, mp 151-153°; $^1$H nmr (deuteriochloroform): δ 3.87 (s, 3H, $OCH_3$), 5.50 (s, 2H, $CH_2$), 6.98 (d, J = 9 Hz, 2H, ArH), 7.25 (s, 2H, $NH_2$), 7.30-7.60 (m, 5H, ArH), 7.82 (d, J = 9 Hz, 2H, ArH), 8.63 (s, 1H, H-6).

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

Preparation of 2-Amino-3-carbamoyl-5-arylpyrazine 1-Oxides **9a-c**.

These compounds were prepared by three methods indicated by A, B or C. The yields of compounds **9a-c** obtained by these methods are summarized in Table 6.

Method A.

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

Method B.

A suspension of 2 mmoles of **7a-c** in 100 ml of dry liquid ammonia was

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

Method C.

A solution of 4 mmoles of **7a**, **8b**, or **8c** in 200 ml of dry 1-propanol saturated with dry ammonia at 0° was stirred at room temperature. After a few hours a bright yellow precipitate appeared. The conversion to **9a-c** was complete after 30 to 36 hours (tlc, solvent B or C). The precipitate was collected; concentration of the filtrate to a volume of 20 ml yielded a second crop. Total yield is given in Table 6. Using aqueous ammonia instead of propanolic ammonia resulted in a very slow conversion to the amide and subsequent hydrolysis to the corresponding acid.

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

This compound was obtained as bright yellow needles, mp 281-283° (mp [17b] 280-282°); $^1$H nmr (90 MHz, DMSO-$d_6$): δ 7.40-7.62 (m, 3H, ArH), 7.91 (br s, 2H, $NH_2$), 8.10-8.27 (m, 2H, ArH), 7.91 and 8.40 (br s, 2H, $CONH_2$), 9.08 (br s, 1H, H-6); ms: m/e 230 ($M^+$, 100), 214 ($M^+$-16, 27).

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

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

This compound was obtained as bright yellow fine needles, mp 274-276°; $^1$H nmr (90 MHz, DMSO-$d_6$): δ 2.33 (s, 3H, $CH_3$), 7.24 (d, J = 7.5 Hz, 2H, ArH), 7.88 (br s, 2H, $NH_2$), 8.07 (d, J = 7.5 Hz, 2H, ArH), 7.88 and 8.39 (br s, 2H, $CONH_2$), 9.03 (s, 1H, H-6); ms: m/e 244 ($M^+$, 100), 228 ($M^+$-16, 79).

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

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

This compound was obtained as long bright yellow needles, mp 254-256°; $^1$H nmr (90 MHz, DMSO-$d_6$): δ 3.80 (s, 3H, $OCH_3$), 6.98 (d, J = 9 Hz, 2H, ArH), 7.82 (br s, 2H, $NH_2$), 8.12 (d, J = 9 Hz, 2H, ArH), 7.82 and 8.40 (br s, 2H, $CONH_2$), 9.01 (s, 1H, H-6); ms: m/e 260 ($M^+$, 100), 244 ($M^+$-16, 59).

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

Preparation of 2-amino-3-carbamoyl-5-arylpyrazines **10a-c**.

Method A. Reduction of **9** with Sodium Dithionite.

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

Method B. Reduction of **9** with Phosphorus Trichloride.

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

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

This compound was obtained in a yield of 97% (method A), 89% (method B), as pale yellow, short needles, mp 239-241° (lit [47] mp 239-240°); $^1$H nmr (90 MHz, DMSO-$d_6$): δ 7.30-7.51 (m, 3H, ArH), 7.63 (br s, 2H, $NH_2$), 8.05-8.20 (m, 2H, ArH), 7.63 and 8.15 (br s, 2H, $CONH_2$), 8.81 (br s, 1H, H-6); ms: m/e 214 ($M^+$).

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

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

This compound was obtained in a yield of 89% (method A), 81% (method B), as pale yellow, short needles, mp 248-250°; $^1$H nmr (90 MHz, DMSO-$d_6$): δ 2.37 (s, 3H, $CH_3$), 7.23 (d, J = 7.5 Hz, 2H, ArH), 7.55 (br s, 2H, $NH_2$), 8.05 (d, J = 7.5 Hz, 2H, ArH), 7.55 and 8.22 (br s, 2H, $CONH_2$), 8.80 (s, 1H, H-6); ms: m/e 228 ($M^+$).

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

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

This compound was obtained in a yield of 86% (method A), 83% (method B), as dark yellow, long needles, mp 210-212°; $^1$H nmr (90 MHz, DMSO-$d_6$): δ 3.80 (s, 3H, $OCH_3$), 6.98 (d, J = 9 Hz, 2H, ArH), 7.56 (br s, 2H, $NH_2$), 8.08 (d, J = 9 Hz, 2H, ArH), 7.56 and 8.24 (br s, 2H, $CONH_2$), 8.76 (s, 1H, H-6); ms: m/e 244 ($M^+$).

*Anal.* Calcd. for $C_{12}H_{12}N_4O_2$ (244.25): C, 59.00; H, 4.95. Found: C, 59.00; H, 4.89.

Preparation of 6-Aryl-4(3*H*)-pteridinone 8-Oxides **11a-c**.

Method A.

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

Method B.

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

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

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

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

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

This compound was obtained in a yield of 57% (method A), 67% (method B); as white needles, mp 322-324° dec; $^1$H nmr (90 MHz, DMSO-$d_6$): δ 2.38 (s, 3H, $CH_3$), 7.35 (d, J = 7.5 Hz, 2H, ArH), 8.07 (d, J = 7.5 Hz, 2H, ArH), 8.24 (s, 1H, H-2), 9.30 (s, 1H, H-7); field desorption ms: m/e 254 ($M^+$).

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

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

This compound was obtained in a yield of 63% (method A), 74% (method B), as pale yellow crystals, mp 325-328° dec; $^1$H nmr (90 MHz, DMSO-$d_6$): δ 3.83 (s, 3H, $OCH_3$), 7.08 (d, J = 9 Hz, 2H, ArH), 8.13 (d, J = 9 Hz, 2H, ArH), 8.22 (s, 1H, H-2), 9.27 (s, 1H, H-7); field desorption ms: m/e 270 ($M^+$).

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

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

Method A.

To a clear yellow solution of 0.14 g of **11a** in 7 ml of 0.5 *M* sodium hy-

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

Method B.

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

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

This compound was obtained in a yield of 77% (method A), 85% (method B), as white short needles, mp 307-309° (lit [13], mp 304° dec); $^1$H nmr (90 MHz, DMSO-$d_6$): δ 7.50-7.66 (m, 3H, ArH), 8.16-8.30 (m, 2H, ArH), 8.35 (s, 1H, H-2), 9.60 (s, 1H, H-7); ms: m/e: 224 ($M^+$, 100), 197 ($M^+$-27, 23), 104 ($M^+$-120, 58).

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

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

This compound was obtained in a yield of 45% (method A), 32% (method B), as white feathery short needles, mp 288-290° (lit [13] mp 286° dec); $^1$H nmr (90 MHz, DMSO-$d_6$): δ 2.38 (s, 3H, $CH_3$), 7.35 (d, J = 7.5 Hz, 2H, ArH), 8.15 (d, J = 7.5 Hz, 2H, ArH), 8.33 (s, 1H, H-2), 9.55 (s, 1H, H-7); ms: m/e 238 ($M^+$, 100), 211 ($M^+$-27, 14), 118 ($M^+$-120, 39).

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

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

This compound was obtained in a yield of 81% (method A), 84% (method B), as yellow short needles, mp 290-292° (lit [13] mp 280° dec); $^1$H nmr (90 MHz, DMSO-$d_6$): δ 3.84 (s, 3H, $OCH_3$), 7.12 (d, J = 9 Hz, 2H, ArH), 8.21 (d, J = 9 Hz, 2H, ArH), 8.28 (s, 1H, H-2), 9.52 (s, 1 H, H-7); ms: m/e 254 ($M^+$, 100), 239 (7), 227 ($M^+$-27, 6), 211 (6), 134 ($M^+$-120, 17), 133 (37).

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

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

To a hot solution of 4,5-diamino-6(1*H*)-pyrimidinone (2.52 g, 20 mmoles, mp 238-240°) [35] in 60 ml of water being brought to *p*H = 7.5 with solid sodium bicarbonate, was added a solution of 25 mmoles of the appropriate arylglyoxal **4a-c** dissolved in 100 ml of ethanol:water (1:1) adjusted to *p*H = 7.5 with aqueous sodium hydroxide. After stirring under gentle reflux for three hours with regular control to keep the *p*H = 7.5, the hot solution is cooled. The (feathery) precipitate was filtered and successively washed with water, ethanol and ether. After drying over phosphorus pentoxide at 100° the yield of the crude product was about 80-90%. The product was recrystallized twice from dimethylsulfoxide [13] at 100°.

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

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

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

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

This compound was obtained in a yield of 38%, as white feathery short needles, mp > 335°; $^1$H nmr (90 MHz, DMSO-$d_6$): δ 2.47 (s, 3H, $CH_3$), 7.43 (d, J = 7.5 Hz, 2H, ArH), 8.17 (d, J = 7.5 Hz, 2H, ArH), 8.30 (s, 1H, H-2), 9.31 (s, 1H, H-6); ms: m/e 238 ($M^+$, 100), 121 (44), 118 ($M^+$-120, 24).

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

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

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

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

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

A mixture of **10a** (0.25 g, 1.1 mmoles), 5 ml of triethyl orthoformate and 5 ml of dimethylsulfoxide was heated under stirring at 110° for 100 hours. To the cooled solution 10 ml of water were added. The precipitate was filtered, washed with water, methanol and ether, and dried to give 0.2 g (64%) of a brown powder. Recrystallization from 1-propanol using charcoal yielded white-greenish fine needles, mp 197-198°; $^1$H nmr (90 MHz, DMSO-$d_6$): δ 2.27 (s, 3H, $CH_3$), 5.23 (s, 2H, $CH_2$), 7.50-7.70 (m, 3H, ArH), 8.15-8.35 (m, 2H, ArH), 8.77 (s, 1 H, H-2), 9.66 (s, 1 H, H-7); ms: m/e 284 ($M^+$, 61), 269 ($M^+$-15, 98), 238 ($M^+$-$CH_2S$, 72), 225 (100); exact mass measurement of $C_{14}H_{12}N_4OS$ ($M^+$ 284.0731 (Theoretical 284.0732).

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

7-Aryl-2,4(1*H*,3*H*)-pteridinediones **14a-c**.

These compounds were prepared according to the method of Pfleiderer and Hutzenlaub [36] given for the preparation of the 7-phenyl derivative. To a solution of (3.57 g, 20 mmoles) of 5,6-diaminouracil hydrochloride acid salt [48] in 60 ml of water, a solution of 25 mmoles of freshly distilled arylglyoxal **4a-c** in 60 ml of ethanol was added. After stirring for 30 minutes at room temperature a yellow precipitate was obtained, which was collected on a Buchner, and washed well with water. The precipitate was dissolved in 500 ml of water (for **14c** 1500 ml was used) with solid potassium hydroxide (*p*H = 11). The solution was refluxed for about seven minutes. After treatment with charcoal and refluxing another five minutes the solution was filtered hot and still hot acidified with glacial acetic acid. After cooling the precipitate was collected by filtration, washed well with water and dried at 120° with phosphorus pentoxide. The products were chromatographically and analytically pure (tlc, solvent B and C).

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

This compound was obtained in a yield of 70%, as bright white chunky crystals, mp >350° (lit [16b,36] mp >350°); $^1$H nmr (90 MHz, DMSO-$d_6$): δ 7.50-7.73 (m, 3H, ArH), 8.10-8.36 (m, 2H, ArH), 9.17 (s, 1 H, H-6); ms: m/e 240 ($M^+$).

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

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

This compound was obtained in a yield of 63%, as off-white chunky

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

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

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

This compound was obtained in a yield of 55%, as yellow chunky crystals, mp >340° dec; $^1$H nmr (90 MHz, DMSO-$d_6$): δ 3.87 (s, 3H, $OCH_3$), 7.12 (d, J = 9 Hz, 2H, ArH), 8.19 (d, J = 9 Hz, 2H, ArH), 9.07 (s, 1H, H-6), 11.58 (br s, 1H, NH), 11.83 (br s, 1H, NH); ms: m/e 270 ($M^+$).

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

Growth of Cells.

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

Preparative Scale Conversion with Xanthine Oxidase (AXO).

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

Immobilization.

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

Oxidation.

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

Kinetic Assays.

The assay for Arthrobacter xanthine oxidase was performed as follows. Aliquots of 2.5 ml of frozen bacterial suspension were disrupted by ultrasonification with a Branson Sonifier B-12 (Branson Sonic Power Company, Danbury, Connecticut) during six times 30 seconds at 36 W in an ice bath, taking care of keeping the temperature below 4°. This solution was centrifuged during 30 minutes at 5000 tpm. This almost clear solution wsa assayed for protein content in duplicate by the method of de Bard and Moss [49] using their modification of the method of Lowry, as well as for enzyme activity in triplicate using 100 μ*M* xanthine and 100 μ*M* 1-methylxanthine at *p*H = 7.2 [50]. For this bacterial enzyme, one unit of enzyme activity is the amount of enzyme which oxidizes 1 μmole of 1-methylxanthine per minute at 25°. The assay conditions were: 100 μ*M* substrate in 50 m*M* potassium phosphate buffer, *p*H = 7.2, including 0.1 m*M* EDTA with the reaction monitored at 292 nm (log Δε = 4.09) for the 1-methylxanthine and at 269 nm (log Δε = 3.89) for xanthine, using oxygen as the final electron acceptor. The rate was determined from the initial slope of the absorbance *versus* time, representing the rate of the disappearance of the substrate.

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

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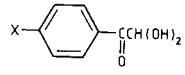

I

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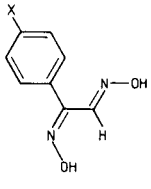
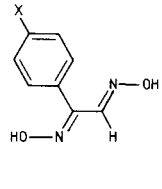


II



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