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Monooxygenation of aromatic compounds by dioxygen with bioinspired systems using non-heme iron catalysts and tetrahydropterins: comparison with other reducing agents and interesting regioselectivity favouring *meta*-hydroxylation

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Abstract—Monooxygenation of aromatic compounds by dioxygen in the presence of catalytic amounts of an iron(II) salt and tetrahydropterins as reducing agents occurs with a regioselectivity favouring *meta*-hydroxylation of arenes bearing an electron-donating substituent, such as anisole, phenetole, toluene, and ethylbenzene. Comparison of similar systems using various reducing agents showed that only tetrahydropterins and ascorbate led to such a major *meta*-hydroxylation. The tetrahydropterin- and ascorbate-dependent systems should be useful for the preparation of *meta*-hydroxylated metabolites of aromatic drugs, as shown here in the case of diclofenac.

1. Introduction

Efficient systems based on iron porphyrins or non-heme iron complexes have been shown to mimic alkene epoxidation and alkane hydroxylation by cytochrome P450 and non-heme dependent monooxygenases.[1–5] Cytochromes P450 and non-heme iron enzymes such as tetrahydrobiopterin (H_4B)-dependent monooxygenases also catalyse the selective hydroxylation of aromatic compounds.[1–6] However, few model systems based on iron catalysts have been described so far for selective and efficient aromatic hydroxylation.[1–5] The most efficient ones use H_2O_2 as oxygen atom donor in the presence of either an iron porphyrin bearing electron-withdrawing β-substituents,[7–9] or a polyazamacrocyclic iron complex, Fe(tris-[*N*-(2-pyridylmethyl)-2-aminoethyl]amine)$(ClO_4)_2$, Fe(TPAA).[10] Many systems using a Fe^{II} salt, O_2 as oxygen atom donor, and a reducing agent, such as ascorbate in the Udenfriend system,[11] have also been shown to be of interest for the hydroxylation of aromatic compounds (see for instance Ref. 12–15).

Tetrahydrobiopterin, H_4B, is a necessary cofactor in non-heme iron aromatic aminoacid monooxygenases,[6] and in NO synthases.[16] In the latter enzymes, it plays a key role by transferring an electron to the heme inside the NOS active site.[17] These recent data led us to investigate the possible specific roles played by tetrahydropterins in monooxygenase model systems using non-heme iron complexes as catalysts, O_2 as a source of oxygen atoms, and a reducing agent. A long time ago, preliminary experiments had shown that a system based on H_4B in the presence of catalytic amounts of $Fe(SO_4)_2(NH_4)_2$ and EDTA was able to hydroxylate the aromatic ring of phenylalanine.[18] Very recently, we have compared the properties of similar systems using different iron catalysts and reducing agents towards the hydroxylation of aromatic compounds. Here, we report that some of these systems using tetrahydropterins are efficient for aromatic hydroxylations, and show an unusual regioselectivity favouring the *meta*-hydroxylation of aromatic compounds bearing an electron-donating substituent.

2. Results and discussion

2.1. Hydroxylation of anisole by a Fe^{II}/EDTA/O_2/tetrahydropterin system

Reaction of anisole with an aerobic phosphate buffer pH 7.4 containing H_4B and catalytic amounts of $Fe(SO_4)_2(NH_4)_2$ and EDTA, for 3 h at 20 °C under conditions similar to those previously used by Viscontini et al. in the case of phenylalanine,[18] led to a mixture of *ortho*-, *meta*- and *para*-methoxyphenols in a very unusual[9,10] 36:46:18 ratio, and to minor amounts of phenol which should come from an

Keywords: Aromatic hydroxylation; Tetrahydrobiopterin; Iron salt; Diclofenac; Ascorbate.
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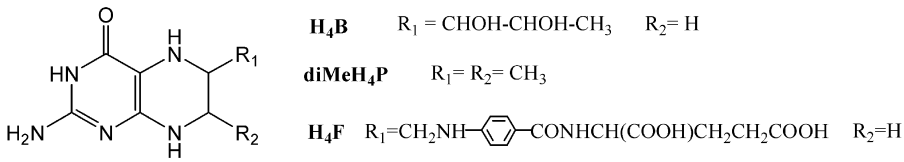
Table 1. Iron-catalyzed hydroxylation of anisole by O_2 and $diMeH_4P$[a]

	Aromatic hydroxylation				Other reaction
	Turnovers/catalyst	Regioselectivity (%)			Phenol (turnovers/catalyst)
		o-OH	*m*-OH	*p*-OH	
Complete system[a]	5.8	30	43	27	0.8
−$Fe^{II}(SO_4)_2(NH_4)_2$	<0.2	—	—	—	<0.2
−$diMeH_4P$	0.3	—	—	—	0.2
−Na borohydride	1.2	35	43	22	0.3
−$diMeH_4P$ – Na borohydride	<0.2	—	—	—	<0.2
−O_2	<0.2	—	—	—	<0.2

[a] Complete system: 1 mL aerobic 0.1 M phosphate buffer pH 7.4, containing $Fe(SO_4)_2(NH_4)_2$, EDTA and $diMeH_4P$ (molar ratio=1:8:16), and 1 mL anisole are vigorously stirred for 2 h at 20 °C; [iron catalyst]=1 mM. Sodium borohydride (10 equiv. relative to $diMeH_4P$) was progressively added during the first hour to the reaction medium in order to regenerate $diMeH_4P$, at least in part. *o*-OH, *m*-OH and *p*-OH are used for *ortho*-, *meta*- and *para*-methoxyphenol.



Scheme 1.

oxidative dealkylation of anisole. Identification of the products was performed by GC and confirmed by GC–MS. Similar results (Table 1) were obtained using the more accessible 6,7-dimethyltetrahydropterin, $diMeH_4P$ (Scheme 1), instead of H_4B. However, replacement of H_4B with dihydrobiopterin, H_2B, led to an inactive system. In their H_4B-containing system, Viscontini et al.[18] have described the beneficial effects on the yields of the addition of a co-reductant, sodium borohydride, which was supposed to regenerate H_4B, at least in part, during the reaction. Table 1 shows that the addition of an excess of sodium borohydride to the $Fe^{II}/EDTA/O_2/diMeH_4P$ system led to a marked increase of the aromatic hydroxylation yield (5.8 turnovers of the iron catalyst instead of 1.2). The absence of dioxygen (identical reaction under anaerobic conditions), or of the catalyst ($Fe(SO_4)_2(NH_4)_2$), or of the reducing agents ($diMeH_4P$+sodium borohydride), led to an almost inactive system (Table 1). Moreover, the absence of $diMeH_4P$ alone led to a dramatic decrease of the yields, indicating that sodium borohydride is almost unable to act as an efficient reducing agent by itself and that its beneficial effects in the complete system is due to its regeneration of $diMeH_4P$ during the reaction.

In Table 1 and the following tables, yields of hydroxylated products are generally expressed in turnovers of the iron catalyst (mol of product formed during the reaction per mol of catalyst). This parameter gives an idea of the catalytic efficiency of the system. From time to time, yields are also expressed in mol of product per mol of starting reducing agent (in %). This second parameter gives the number of electrons used by the system for dioxygen activation and hydroxylation of the aromatic substrates. It is an index of the decoupling between electron consumption by the system and hydroxylation of the substrate. In most bioinspired systems using an iron catalyst, O_2 and a reducing agent, this decoupling is large and yields based on the reducing agent are low.[15] In the system using both $diMeH_4P$ and sodium borohydride (in order to regenerate $diMeH_4P$), yields are expressed relative to starting $diMeH_4P$, simply because sodium borohydride rapidly reacts with water and only a small, non measurable part of it is used to regenerate $diMeH_4P$.

Kinetic experiments on the oxidation of anisole by the $Fe^{II}/EDTA/O_2/diMeH_4P/NaBH_4$ system showed that the reaction was almost complete after 2 h, with an initial rate of methoxyphenols formation of 3.5 turnovers of the iron catalyst per hour (Fig. 1). Stopping of the reaction was presumably due to the consumption of the reducing agents, as a further addition of $diMeH_4P$ and sodium borohydride led to similar yields and rates. Figure 1 shows that the regioselectivity of anisole hydroxylation varies as a function of the reaction time. At the beginning of the reaction (~1 h), the relative importance of *meta*-hydroxylation is at its maximum level, with an *ortho:meta:para* ratio of about 25:50:25, whereas this ratio tends towards 34:36:30 at the end of the reaction. This could mean that *meta* hydroxylation of anisole is favoured in the presence of the high concentrations of reductant that occur at the beginning of the reaction, and that could keep the Fe(II) concentration high.

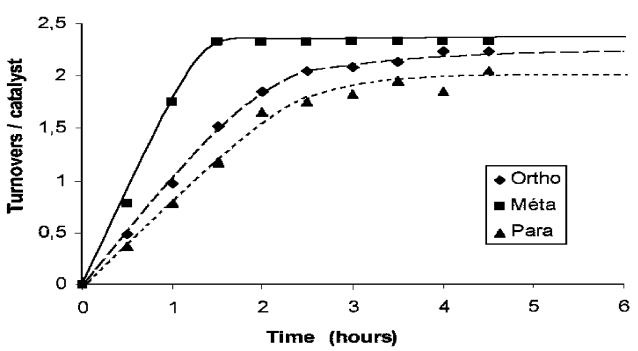


Figure 1. Kinetic study of the formation of *o*-, *m*- and *p*-methoxyphenols upon oxidation of anisole by the $Fe^{II}/EDTA/diMeH_4P/NaBH_4$ system (conditions of Table 1).

Table 2. Hydroxylation of anisole by O_2 and diMeH_4P in the presence of $Fe(SO_4)_2(NH_4)_2$ and various chelates[a]

Chelate	Aromatic hydroxylation				
	Total turnovers/catalyst	Total yield/diMeH_4P (%)	Regioselectivity (%)		
			o-OH	*m*-OH	*p*-OH
Without EDTA	<0.2	<1	—	—	—
EDTA	5.8	37	30	43	27
EGTA	0.2	1	45	45	10
EDDA	3.2	20	30	45	25
DTPA	1.5	9.5	47	32	21
HETA	2.9	18	33	39	28

[a] Conditions as in the complete system described in Table 1.

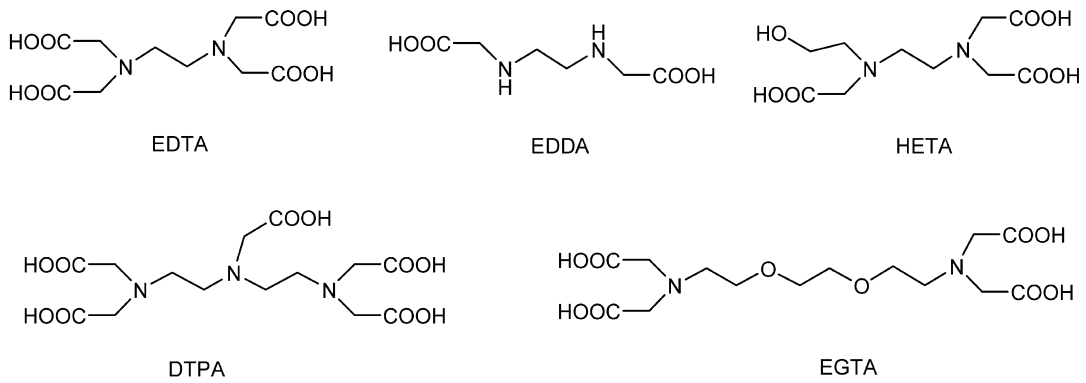


Figure 2. Formula of the various chelates used in this study.

2.2. Studies of the reaction parameters

Table 2 shows the importance of the EDTA ligand on anisole hydroxylation by the above mentioned system. In the absence of EDTA, the system was almost unable to catalyze this reaction. Replacement of EDTA with analogous polydentate ligands, such as EGTA, EDDA, DTPA and HETA[†] (Fig. 2) led to a marked decrease of the methoxyphenols yields (0.2–3.2 turnovers/iron instead of 5.8 in the case of EDTA). This decrease of the yields was accompanied by changes of the regioselectivity which was still in favour of *meta*-methoxyphenol except in the case of DTPA. The distances between the coordinating groups of EDTA seem to play a crucial role, since replacement of EDTA with EGTA, which has a longer chain led to a dramatic decrease of the yields (Table 2).

A study of the effects of the Fe^{II}/EDTA molar ratio on anisole hydroxylation showed that the best compromise for reaching good hydroxylation yields and a regioselectivity in favour of *meta*-hydroxylation was obtained with a 1:10 ratio (data not shown).

Replacement of $Fe(SO_4)_2(NH_4)_2$ with the various non-heme iron complexes, $[(TPAA)Fe^{II}](ClO_4)_2$,[10] $(L^2_5FeCl)PF_6$ [19] and $[Fe(TPA)(CH_3CN)_2](ClO_4)_2$,[20] or with iron porphyrin complexes, such as Fe(III)[tetra-pentafluorophenyl-β-tetra-sulfonato-porphyrin][21] and microperoxidase, MP11Fe(III),[22] failed to yield significant amounts of methoxyphenols.

Moreover, a study of the effects of the concentration of the iron salt showed us that the yield of *meta*-methoxyphenol increased with the Fe^{II} salt concentration up to 0.5–1 mM (Table 3). For concentrations higher than 1 mM, the yield of anisole hydroxylation dramatically decreased. Therefore, a Fe^{II} concentration of 1 mM was used in the following studies.

Table 3. Effects of Fe^{II} concentration on iron-catalyzed hydroxylation of anisole by O_2 and diMeH_4 P[a]

$[Fe^{II}]$ (M)	Aromatic hydroxylation			
	Total turnovers/catalyst	Regioselectivity (%)		
		o-OH	*m*-OH	*p*-OH
4×10^{-5}	0.8	80	10	10
5×10^{-4}	6.9	39	30	31
10^{-3}	5.8	30	43	27
2×10^{-3}	0.7	29	43	28
4×10^{-3}	0.2	50	25	25

[a] Conditions as in the complete system described in Table 1; molar ratio 1:8:16:160 for Fe^{II}/EDTA/diMeH_4P/sodium borohydride was conserved.

Finally, a study of the effects of pH on the hydroxylation of anisole showed that the total yield of methoxyphenols reach a maximum value at pH 7.4 (5.8 turnovers) (Table 4). However, the relative importance of *meta*-hydroxylation was enhanced when pH was increased from 4.5 up to 9, and was maximum at pH 9 (52% of total methoxyphenols). The

[†] EDTA, EGTA, EDDA, DTPA and HETA correspond to ethylenediamine tetraacetic acid, ethylenebis(oxyethylenenitrilo)-tetraacetic acid, ethylenediamine-*N*,*N*′-diacetic acid, diethylenetriamine pentaacetic acid and *N*(2-hydroxyethyl)ethylenediaminetriacetic acid respectively (see Fig. 2).

Table 4. Effects of pH on iron(II)-catalyzed hydroxylation of anisole by O_2 and diMeH$_4$ P[a]

pH	Aromatic hydroxylation			
	Total turnover/catalyst	Regioselectivity (%)		
		o-OH	*m*-OH	*p*-OH
4.5	1.1	54	31	15
6	5.1	44	34	22
7.4	5.8	30	43	27
8.2	3.4	35	42	23
9	2.5	29	52	19
11.8	1.8	39	39	22

[a] Conditions as in the complete system described in Table 1, except for the buffer which was adapted to each pH (see Section 4).

following experiments were performed at pH 7.4 that was a good compromise for good yields and regioselectivity.

2.3. Generalization to various substrates

The Fe$(SO_4)_2(NH_4)_2$/EDTA/O_2/diMeH$_4$P system was also found to hydroxylate other aromatic compounds, such as benzene itself, with formation of the corresponding phenols (total turnovers of catalyst for aromatic hydroxylation between 1 and 5.8, and yields based on diMeH$_4$P between 6.5 and 37%) (Table 5). The most distinctive and surprising characteristic of this system is the unusual regioselectivity of its hydroxylation of aromatic compounds bearing an electron-donating substituent. Thus, hydroxylation of anisole mainly occurred at the *meta* position (43% of total aromatic hydroxylation), and *meta*-hydroxylation of ethoxybenzene reached 38% of total aromatic hydroxylation. In contrast, the efficient iron catalyst/H_2O_2 systems described previously[9,10] almost exclusively led to *ortho*- and *para*-hydroxylation of anisole and ethoxybenzene (*meta*-hydroxylation <2% of total aromatic hydroxylation), as expected for systems involving electrophilic hydroxylating species. With the present system, hydroxylation of the aromatic ring of toluene and ethylbenzene also occurred mainly at the *meta* position (50% of total aromatic hydroxylation, Table 5), whereas the previous iron catalyst/H_2O_2 systems[7–10] gave much lower levels of *meta*-hydroxylation for these two substrates (between 11 and 25%). This unusual regioselectivity of the diMeH$_4$P-dependent system was particularly well illustrated in the case of diclofenac (Scheme 2). This anti-inflammatory drug is metabolized by human cytochromes P450 with major formation of 4′-hydroxydiclofenac and minor formation of 5-hydroxydiclofenac, which are both derived from hydroxylations at the *para* positions of the strong electron-donating NH group.[23,24] Iron porphyrin–H_2O_2 (or tBuOOH) systems mainly hydroxylate diclofenac at position 5, 4′-hydroxydiclofenac being formed in small amounts.[23] In contrast, the diMeH$_4$P-dependent system led to significant amounts of 3′-hydroxydiclofenac, which results from *meta*-hydroxylation of the dichlorophenyl ring of diclofenac (32% of total aromatic hydroxylation, Table 5). This 3′-hydroxy metabolite, which was only formed in trace amounts with cytochromes P450[24] and iron porphyrin model systems,[23] was produced by the diMeH$_4$P-dependent system in amounts comparable to those of 4′- and 5-hydroxydiclofenac (Table 5).

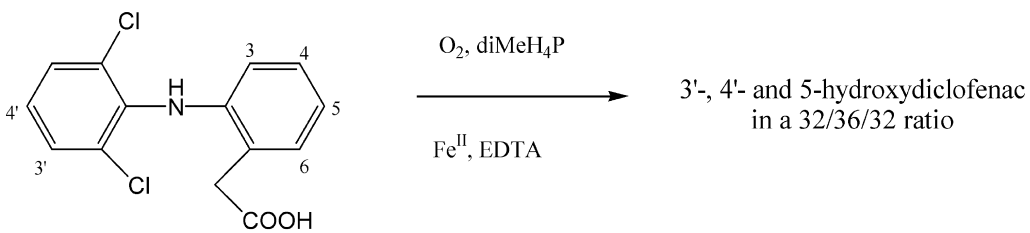
2.4. Effects of the replacement of diMeH$_4$P by other reducing agents

In order to better understand the origin of the important

Table 5. Regioselectivity of the hydroxylation of aromatic compounds by O_2 and diMeH$_4$P in the presence of Fe$(SO_4)_2(NH_4)_2$ and EDTA[a]

Substrate	Aromatic hydroxylation					Other reactions yields (% based on diMeH$_4$P)[b,c]
	Total turnovers/catalyst	Total yield (% based on diMeH$_4$P)[b]	Regioselectivity (%)			
			o-OH	*m*-OH	*p*-OH	
Anisole	5.8	37	30	43	27	5 (phenol)
Ethoxybenzene	4	25	33	38	29	4 (phenol)
Toluene	2.8	17.5	40	50	10	3 ($PhCH_2OH$)
Ethylbenzene	1	6.5	40	50	10	74 ($PhCOCH_3$)+17 ($PhCHOHCH_3$)
Benzene	5	31				
Diclofenac	2.5	15.5	32 (3′-OH): 36 (4′-OH): 32 (5-OH)			

[a] Conditions as in the complete system described in Table 1. In the case of diclofenac, saturating amounts of diclofenac sodium salt were dissolved in the phosphate buffer medium.
[b] Yields based on starting diMeH$_4$P; one turnover of the catalyst roughly corresponds to 6% yield.
[c] Reactions occurring in competition with aromatic hydroxylation are oxidative dealkylation for anisole and ethoxybenzene, and benzylic hydroxylation in the case of toluene and ethylbenzene.



Scheme 2.

Table 6. Effects of the nature of the reducing agent on iron-catalyzed hydroxylation of anisole by O_2[a]

Reducing agent	Aromatic hydroxylation				
	Total turnovers/catalyst	Total Yield[b]/reductant (% based)	Regioselectivity (%)		
			o-OH	*m*-OH	*p*-OH
$DiMeH_4P+NaBH_4$	5.8	37 (3.3)	30	43	27
$DiMeH_4P$	1.2	7.5	35	43	22
H_4B	0.9	5.5	36	46	18
H_4F	1.5	9	39	33	28
Ascorbate	6.4	40	31	40	29
Catechol	0.7	4.5	44	—	56
Hydroquinone	0.3	2	58	—	42
Trimethylhydroquinone	2.9	17.5	39	22	39
Thiophenol	<0.2	<1	—	—	—
α-Naphthol	<0.2	<1	—	—	—
Methylhydrazine	0.9	5.5	36	18	46
2-Mercaptobenzoic acid	<0.2	<1	—	—	—
Hydrazobenzene	<0.2	<1	—	—	—
NADH	<0.2	<1	—	—	—

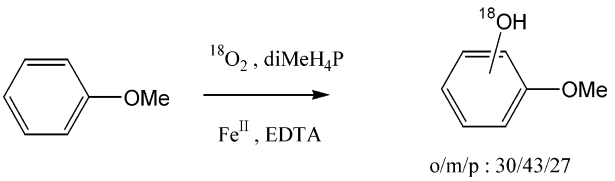
[a] Conditions as in the complete system described in Table 1, but without addition of sodium borohydride (except as specified for the first line of the table). Iron catalyst/reductant molar ratio=1/16.

[b] Yields are based on the starting reducing agent; in the case of $diMeH_4P$+Na borohydride, an excess of sodium borohydride was also used and yields are expressed on the basis of starting $diMeH_4P$. Yield in parentheses is based on starting $diMeH_4P$+sodium borohydride.

meta-hydroxylation observed with the Fe^{II}/EDTA/O_2/$diMeH_4P$ system, we have replaced $diMeH_4P$ by various reductants. Some of them have been previously used in oxidizing systems based on an iron catalyst, O_2 and a reducing agent. This is the case of catechol,[25] hydroquinone,[26] trimethylhydroquinone,[27] 2-mercaptobenzoic acid,[28] hydrazobenzene[29] and ascorbic acid.[11] Table 6 shows that the regioselectivities the most in favour of *meta*-hydroxylation of anisole were obtained with tetrahydropterins $diMeH_4P$ and H_4B, and with ascorbate. Only these three reducing agents led to a major formation of *meta*-methoxyphenol. Tetrahydrofolate, H_4F (Scheme 1), another natural tetrahydropterin cofactor, gave results similar to H_4B and $diMeH_4P$, however with lower relative amounts of *meta*-methoxyphenol. Trimethylhydroquinone and methylhydrazine also led to significant amounts of *meta*-methoxyphenol but as a minor product when compared to its *ortho* and *para* isomers. Catechol and hydroquinone exclusively led to *ortho*- and *para*-methoxyphenol. All the other tested reducing agents, thiophenol, α-naphthol, 2-mercaptobenzoic acid, hydrazobenzene and NADH failed to produce methoxyphenols in significant amounts. These data clearly showed that the structure of the reducing agent is a key factor in aromatic hydroxylations by the Fe^{II}/EDTA/O_2/reductant systems. Two reducing agents emerge from this comparison, $diMeH_4P$ (or H_4B) and ascorbate; they are much superior to the others both in terms of catalytic efficiency for anisole hydroxylation, with about six turnovers of the catalyst, and in terms of regioselectivity in favour of *meta*-hydroxylation (43 and 40% of total aromatic hydroxylation).

2.5. Complementary mechanistic experiments

2.5.1. Origin of the oxygen atom inserted into substrates. Oxidation of anisole by $^{18}O_2$ (containing 98% ^{18}O) and the Fe^{II}/EDTA/$diMeH_4P$ system under conditions similar to those of Table 1 (see Section 4) led to *ortho*-, *meta*- and *para*-methoxyphenols bearing a phenolic oxygen atom containing 98±1% of ^{18}O isotope, as shown by mass spectrometry coupled to gaz chromatography. When the same reaction was performed with $^{16}O_2$ in $H_2^{18}O$ (98% enriched), the methoxyphenol products contained less than 1% ^{18}O. These data clearly show that the oxygen atom incorporated into anisole almost exclusively came from O_2 (Scheme 3), whatever the position, *ortho*, *meta* or *para*, of the hydroxyl group.



Scheme 3.

2.5.2. Possible role of H_2O_2. All the iron/O_2/reductant model systems of monooxygenases led to a partial decoupling between the consumption of electrons from the reducing agent and the monooxygenase reaction, which results in the formation of products coming from dioxygen reduction such as H_2O_2. In order to evaluate the role of H_2O_2 possibly produced during the studied reactions, oxidation of anisole by the Fe^{II}/EDTA/O_2/$diMeH_4P$ system was performed in the presence of catalase. No significant difference was noted in the yields observed with or without catalase, implying that H_2O_2 does not play an important role in these reactions.

2.6. Discussion

Our data show that systems based on tetrahydropterins as reducing agents, in the presence of a catalytic amount of an iron(II) salt and EDTA, which are closely related to the system previously used by Viscontini et al. for the hydroxylation of phenylalanine,[18] also catalyse the hydroxylation of many aromatic compounds such as benzene, anisole, ethoxybenzene, toluene, ethylbenzene and diclofenac. The main characteristic of these systems is their regioselectivity in favour of the *meta*-hydroxylation of

aromatic compounds bearing an electron-donating substituent. A comparative study of many systems using other reducing agents, under identical conditions, clearly showed that only diMeH$_4$P (or other tetrahydropterins) and ascorbate led to such a regioselectivity in favour of *meta*-hydroxylation. This suggests that a reinvestigation of *meta*-hydroxylation by Udenfriend-type systems (Fe^{II}/O_2/ascorbate) should be very much interesting. In fact, there are few precedents of such iron-catalyzed *meta*-monooxygenations of aromatic compounds bearing an electron-donating substituent in the literature (for reviews, see Ref. 12–14). Most of the previously reported iron-catalyzed oxidations of such aromatic compounds, using either H_2O_2,[7–10,13,15] or O_2 and a reductant,[12–15,30] led to preferential *ortho*- and *para*-hydroxylations. Many reports on systems related to the one originally described by Udenfriend et al., and using O_2 and ascorbate, have been published; however very few mentioned *meta*-hydroxylation. Hamilton et al. have used a $FeClO_4$/EDTA/O_2/ascorbate system and found at best 18% of *meta*-methoxyphenol (relative to total aromatic hydroxylation) in the hydroxylation of anisole.[31] At the same period, Norman and Lindsay Smith[13] have used a similar system and reported regioselectivities for anisole and toluene involving 22 and 25% of *meta*-hydroxylated compound, respectively. More data have been published on *meta*-hydroxylation of aromatic compounds bearing an electron-donating substituent by another class of systems using Fe^{II} or other reduced metal ions and dioxygen without any reducing agent.[13,14,32–34] These systems are not catalytic and yields are expressed relative to the metal ion. In the following, we will only consider the systems using a Fe^{II} salt[13,32–34] for sake of comparison with our results exclusively based on Fe^{II} catalysts. It has been noticed that the relative amounts of the *meta*-hydroxylated products increased when the iron concentration increased, and the regioselectivity the most in favour of *meta*-hydroxylation of anisole observed by Dearden et al.[34] corresponded to a 39:50:11 *o:m:p* methoxyphenols ratio. These authors also mentioned that when they added *N*-benzyl-1,4-dihydronicotinamide as a reducing agent to their system, the *meta*-hydroxylated product almost completely disappeared.[34]

The mechanisms of such *meta*-monooxygenations of aromatic compounds are presently almost unknown. The involvement of electrophilic species such as Fe^{III}–OOH or high-valent iron–oxo intermediates, that are usually proposed in iron complex/H_2O_2 systems, is unlikely, because these systems have been shown to mainly lead to *ortho*- and *para*-hydroxylated products.[7–10,13,15] *Meta*-hydroxylation does not seem to be due to the ·OH radical, because the regioselectivity observed for anisole hydroxylation by ·OH appears to correspond to a 84:0:16 molar ratio of the *o*-, *m*-, *p*-methoxyphenols.[13]

The involvement of a polymetallic complex, containing at least two iron atoms, Fe–O and/or Fe–OO moieties, and the reducing agent, that would first attack the electronically favoured *para* (or *ortho*) position of the aromatic substrate and then its *meta*-position, would be at least partly in agreement with previous proposals from the literature.[13,34] This would also fit with the increase of *meta*-hydroxylation upon increasing of the iron concentration, as previously reported in some articles.[13,34] In that regard, it is noteworthy that polymetallic iron complexes are formed upon reaction of Fe^{II} salts with EDTA,[35] and that some diiron complexes are able to catalyze the oxidation of toluene to *meta*-cresol, although in very different conditions.[36] The reasons why only two of the tested reducing agents, diMeH$_4$P (or H$_4$B) and ascorbate, lead to a major *meta*-hydroxylation of anisole (Table 6), remain to be determined.

3. Conclusion

Whatever its mechanism may be, the iron/EDTA/tetrahydropterin/O_2 system is interesting because of its unusual regioselectivity favouring the incorporation of an oxygen atom from O_2 at the *meta*-position of aromatic compounds bearing an electron-donating substituent. Our results show that this is true for many of such aromatic compounds. They also show that only two reducing agents, diMeH$_4$P and ascorbate, used in the various Fe^{II}/EDTA/reductant/O_2 systems that we have compared, lead to this major *meta*-hydroxylation of anisole. Interestingly, the two corresponding systems also lead to the most efficient catalysis of anisole hydroxylation (Table 6).

Since a fast access to small amounts of various hydroxylated derivatives of drugs is required for identification of drug metabolites and for a first evaluation of the pharmacological properties of these metabolites, the aforementioned diMeH$_4$P- and ascorbate-dependent systems should be useful for the preparation of *meta*-hydroxylated metabolites of aromatic drugs, as illustrated above in the case of diclofenac.

4. Experimental

4.1. Reactants

All the reactants and products were commercially available and purchased from Acros, Sigma, Aldrich and Alfa Aesar (iron salt). They were used without further purification except for the aromatic compounds that were eluted on a small alumina column prior to use. $H_2^{18}O$ (98% enriched in ^{18}O) and $^{18}O_2$ (98% enriched in ^{18}O) were purchased from EURISO-TOP (Saclay, France).

4.2. General procedure

Hydroxylation of aromatic substrates was performed at room temperature in vials equipped with a magnetic stirrer. The organic phase consisting of 1 mL of aromatic substrate (anisole, phenetole, toluene, ethylbenzene or benzene) was added to 1 mL of 0.1 M phosphate buffer, pH 7.4, containing $Fe(SO_4)_2(NH_4)_2{\cdot}6H_2O$ (10^{-3} M, 10^{-6} mol, 0.4 mg), EDTA (8×10^{-3} M, 8×10^{-6} mol, 2.3 mg), diMeH$_4$P (1.6×10^{-2} M, 1.6×10^{-5} mol, 3.8 mg) and $NaBH_4$ (1.6×10^{-4} mol, 6.4 mg), the latter being progressively added in one hour. When other reductants than diMeH$_4$P were used, 1.6×10^{-5} mol was also introduced, but no sodium borohydride was added. After 2–3 h stirring at 20 °C, an internal standard ($PhCOCH_3$ or PhI,

1.6×10^{-5} mol) was added and the reaction mixture was analyzed by gas chromatography.

A strictly similar procedure was followed when pH buffer was modified. In those cases, we used an acetate buffer adjusted to pH=4.5, a citric acid/Na_2HPO_4 buffer pH=6, a trismabase/HCl buffer pH=8.2, a glycine/NaOH buffer pH=9 and a Na_2HPO_4/NaOH buffer pH=11.8.

Reactions under anaerobic conditions were done by 'freeze–thaw cycles' of a vial containing all the reactants except the reducing agent, and of a second vial containing the reducing agent solution under argon. The content of the first vial was then transferred onto the reductant solution under argon.

4.3. Hydroxylation of diclofenac

The aqueous phase (1 mL) contained $Fe(SO_4)_2(NH_4)_2{\cdot}6H_2O$ (10^{-3} M), and EDTA (8×10^{-3} M), and 100 mg of the sodium salt of diclofenac were added to the solution. After 2–3 h stirring at 20 °C, samples were centrifuged to precipitate excess diclofenac, and products were analyzed by reverse phase HPLC, as described below.

4.4. Oxidation of anisole using $^{18}O_2$

500 μL of 0.1 M phosphate buffer, containing 7.0×10^{-7} mol of $Fe(SO_4)_2(NH_4)_2$, 5.6×10^{-6} mol of EDTA, and 1 mL of anisole were deaerated by three freeze–thaw cycles. 1.1×10^{-5} mol of diMeH$_4$P were dissolved in 200 μL of 0.1 M phosphate buffer (pH 7.4) and deaerated by the same procedure. $^{18}O_2$ was introduced in the main vial and diMeH$_4$P was added using a deoxygenated syringe. After 2–3 h stirring, products were analysed by GC–MS as described below.

4.5. Oxidation of anisole using $H_2^{18}O$

200 μL of 0.1 M phosphate buffer (pH 7.4) containing $Fe(SO_4)_2(NH_4)_2$ (10^{-3} M, 2×10^{-7} mol) and EDTA (8×10^{-3} M, 1.6×10^{-6} mol) were lyophilized and re-dissolved in 200 μL of $H_2^{18}O$. 500 μL of anisole was added in the vial. diMeH$_4$P (3.2×10^{-6} mol) was introduced in the solid state, followed by $NaBH_4$ (1.6×10^{-1} M, 3.2×10^{-5} mol), added in four times. After 2–3 h, products were analysed by GC–MS as in $^{18}O_2$ experiments.

4.6. Product analysis and identification

GC analyses were done using either a packed 5% FFAP (polar) column for anisole and benzene, or a capillary BP20 (polar) column for toluene and ethylbenzene, with detection with a flame ionization detector (FID). The products formed were analyzed by comparison of their retention time with those of authentic samples and by gas chromatography-mass spectrometry analysis using a Hewlett-Packard 5890 Series II GC coupled with a HP5972 mass selective detector.

HPLC analyses of diclofenac metabolites were done using a Chromatem 380 apparatus. Supernatant aliquots were injected onto a X-terra MS C18 column (3.0×150 mm, 5 μm). The mobile phase (20 mM phosphate buffer (pH 8)/[acetonitrile/water (90/10)], gradient 20% up to 50% in 27 min) was delivered at a rate of 0.6 mL/min. Monitoring of the column effluent was performed with a detector at 270 and 280 nm. The reaction products were compared to authentic samples of 3′-, 4′- and 5-hydroxydiclofenac kindly provided by Ciba-Geigy (Basel, Switzerland).

References and notes

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