Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2012, **10**, 4554

www.rsc.org/obc

PAPER

Biomimetic oxidation of aromatic xenobiotics: synthesis of the phenolic metabolites from the anti-HIV drug efavirenz

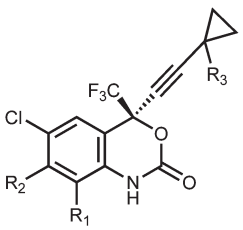
Riccardo Wanke,* David A. Novais, Shrika G. Harjivan, M. Matilde Marques and Alexandra M. M. Antunes *

Received 27th January 2012, Accepted 5th April 2012
DOI: 10.1039/c2ob25212k

We report the oxidation of the first line anti-HIV drug efavirenz (EFV), mediated by a bio-inspired nonheme Fe-complex. Depending upon the experimental conditions this system can be tuned either to yield the major EFV metabolite, 8-hydroxy-EFV, in enantiomerically pure form or to mimic cytochrome P450 (CYP) activity, yielding 8-hydroxy-EFV and 7-hydroxy-EFV, the two phenolic EFV metabolites reported to be formed *in vivo*. The successful oxidation of the anti-estrogen tamoxifen and the equine estrogen equilin into their CYP-mediated metabolites supports the general application of bio-inspired nonheme Fe-complexes in mirroring CYP activity.

Introduction

Efavirenz [EFV, (*S*)-6-chloro-4-(cyclopropylethynyl)-4-(trifluoromethyl)-1,4-dihydro-2*H*-3,1-benzoxazin-2-one, Scheme 1] is a non-nucleoside reverse transcriptase inhibitor (NNRTI) administered as a first-line treatment against HIV.[1] Despite its efficacy, a major shortcoming of EFV use is its association with neurotoxic and hepatotoxic events.[2–4] Although the reasons for the drug's adverse effects are currently not clear, there is evidence that metabolic activation to reactive electrophiles capable of reacting with bionucleophiles leads to the formation of covalent adducts that are involved in the initiation of species-specific toxic outcomes.[5] EFV is extensively metabolized by cytochrome P450 (CYP), undergoing primary oxidation on the aromatic ring to the phenolic products 7-OH-EFV (minor) and 8-OH-EFV (major), and secondary oxidation on the cyclopropane ring (at C14) to 8,14-diOH-EFV (Scheme 1).[6] Very recently, an *in vitro* study using primary human hepatocytes revealed that 8-OH-EFV was a more potent modulator of hepatic cell death than the parent compound, suggesting that this primary metabolite could potentially contribute to EFV-mediated hepatotoxicity.[7] This correlation between 8-OH-EFV and toxicity may be very significant, due to the potentially high *in vivo* systemic exposure to this metabolite.[8] Moreover, given that EFV increases the mRNA and protein expression of CYP2B6,[9–11] chronic EFV use results in increased formation of 8-OH-EFV. Indeed, in patients with the CYP2B6*1/1 genotype the EFV/8-OH-EFV ratio reaches approximately 0.98 and 0.74 after 4 weeks and 16 weeks of treatment, respectively,[8] and over a period of time the concentration of the metabolite can exceed that of the parent compound. In order to assess unequivocally the role of metabolic EFV activation in the initiation of toxic events elicited by chronic use of the drug, the availability of considerable amounts of enantiomerically pure EFV metabolites is essential, so as to enable *in vitro* and *in vivo* toxicological studies.



R_1=R_2=R_3 = H; **EFV**
R_1 = OH, R_2= R_3 = H; **8-OH-EFV**
R_2 = OH, R_1=R_3 = H; **7-OH-EFV**
R_1=R_3 = OH, R_2 = H; **8,14-diOH-EFV**

Scheme 1 Structures of efavirenz (EFV) and its metabolites: 8-OH-EFV, 7-OH-EFV, and 8,14-diOH-EFV.

The syntheses of the racemic Phase I EFV metabolites have been described;[12,13] however, the low efficiency of the multistep synthetic pathways precludes large scale preparations of the pure enantiomers. Particularly, the racemate of the major EFV metabolite, 8-OH-EFV, is obtained after nine synthetic steps in 29% yield; following chiral HPLC separation the enantiomerically pure product is obtained in 14% overall yield.[13] This limitation motivated us to investigate new synthetic strategies in order to simplify the procedure and maximize the yield of the enantiomerically pure metabolite. Despite the significant challenge, we considered the feasibility of a biomimetic direct oxidation of

Centro de Química Estrutural, Instituto Superior Técnico, Universidade Técnica de Lisboa, 1049-001 Lisboa, Portugal. E-mail: alexandra.antunes@ist.utl.pt, riccardowanke@ist.utl.pt; Fax: +351-21-8464455; Tel: +351-21-8417627, +351-21-8419388

EFV into its Phase I metabolites since the success of this strategy could drastically change the common approach of research in this field. Among the large variety of 'bio-inspired' catalysts that mimic the versatile CYP mechanisms, the class of nonheme iron-based complexes constitutes one of the most explored groups of catalysts for the oxidation of different substrates, including the hydroxylation of alkanes, alkenes and aromatic compounds and the epoxidation of olefins.[14–17] In particular, the N_4-tetradentate Fe^{II} family of compounds (*e.g.*, [Fe^{II}(bpmen)$(OTf)_2$], bpmen: *N*,*N*′-bis-(2-pyridylmethyl)-1,2-diaminoethane) is reported[18–20] to pass through the formation of a high valence Fe^{V}=O species, suggesting that it is capable of accessing the formal Fe^{V} oxidation state commonly associated with the active oxidant in CYP and other heme enzymes.[21,22] Therefore, we explored this possibility and report herein the development of a method for direct aromatic hydroxylation of EFV and other xenobiotics, consisting on the use of the easily accessible [Fe^{II}(bpmen)$(OTf)_2$]–H_2O_2 oxidant system in the presence of acetic acid.

Results and discussion

The establishment of a procedure for the preparation of significant amounts of 8-OH-EFV from direct oxidation of EFV was developed considering the best yields, combined with the cheapest, easiest and cleanest processes, in order to extend this new methodology to large scale preparations. To select the most effective system, screening reactions were conducted under standard conditions,[23,24] using an acetonitrile (AcCN) solution of EFV, 10 mol% of catalyst, and hydrogen peroxide (0.45 eq.) in the presence of acetic acid. The yields were calculated from the HPLC peak areas, corrected for the corresponding molar absorptivity at the monitored wavelength (254 nm). Among the tested catalysts (Table 1) were [Fe(bpmen)$(OTf)_2$][25,26] (**1**), [Fe(*S*,*S*-PDP)$(NCCH_3)_2$] $(SbF_6)_2$ [23] (**2**, *S*,*S*-PDP: 2-({(*S*)-2-[(*S*)-1-(pyridin-2-ylmethyl)pyrrolidin-2-yl]pyrrolidin-1-yl}methyl)pyridine), [$FeCl_2(Py_2S_2)$][24] (**3**, Py_2S_2: 1,6-bis(2′-pyridyl)-2,5-dithiahexane) and [Cu(bpmen)$(OTf)_2$] (**4**). Complexes **3** and **4** displayed limited activity (Table 1, entries 29 and 30), while both the use of a simple Fe^{II} salt (*i.e.*, $Fe(OTf)_2$, Table 1, entry 31) and the absence of catalyst (Table 1, entry 32) were completely ineffective. Of all the assessed catalysts, those containing the N_4–Fe^{II} core appeared to be the most efficient. Although catalysts **1** and **2** had comparable efficiencies (Table 1, entries 2 and 28), the first was chosen for subsequent reactions due to the availability of an easy and cost-efficient method for its preparation,[25,26] contrasting with the high cost of the commercially available **2**. It is noteworthy that whereas 8-OH-EFV was formed in moderate yield under Fenton's oxidation conditions (Table 1, entry 33), the complexity of the reaction mixture clearly indicated non-selective oxidation and precluded a synthetic scale-up. Additional studies were carried out towards process optimization, using **1** as the selected catalyst.

Although an increment in the number of hydrogen peroxide equivalents from 0.45 to 1.35 (Table 1, entries 2 *vs.* 23) led to *ca.* 1.6-fold increase in the yield of 8-OH-EFV, a portionwise addition[27] of the same amount of oxidant, over an extended period of time, resulted in a lower efficiency (Table 1, entry 5), presumably due to 8-OH-EFV degradation into further oxidized derivatives. The use of an alternative peroxide such as *m*-chloroperbenzoic acid (*m*-CPBA, Table 1, entry 26) provided a lower, though still significant, yield. However, when *t*BuOOH was tested, even under microwave irradiation and/or high temperature (data not shown) no reaction occurred, plausibly due to steric hindrance reasons. Similarly to what has been reported for other substrates,[23,24] the use of an acid, as co-catalyst (co-CAT), improved the oxidation efficiency substantially (Table 1, entries 1 and 2 *vs.* 27). Since the nature of the adequate acid is highly related with the catalytic system,[25,28] different acids were screened (*e.g.*, nitric acid, pyrazine-2-carboxylic acid, acetic acid; results not shown); of these, acetic acid led to cleaner reaction mixtures and the best yields. The addition of ethyl acetate (AcOEt) as co-solvent (up to 50% v/v) significantly enhanced the yield, probably due to increased EFV solubility (Table 1, entries 2 *vs.* 3 and 23 *vs.* 22). However, using higher percentages of AcOEt resulted in lower reaction efficiencies (Table 1, entries 19 and 20 *vs.* 14 and 15 *vs.* 12 and 13), probably due to decreased solubility of **1**.

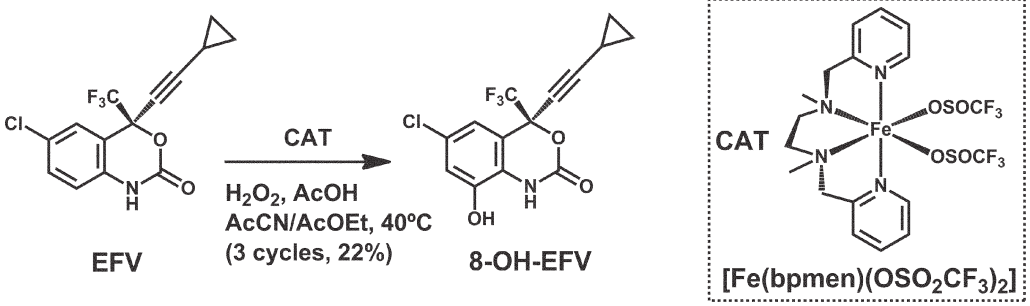
Whereas these types of complexes are reported to react very fast with H_2O_2,[29] for this system we observed an yield increment with increasing reaction times (Table 1, entries 18 *vs.* 19 *vs.* 20, *cf.* Experimental section). Likewise, better yields were obtained when reactions were conducted at 40 °C (Table 1, entries 19 and 20 *vs.* 16 and 17), albeit this type of transformations are usually conducted at room temperature.[29]

Arene oxidations by these systems tend to result in Fe(III) bound phenolate complexes, that block catalytic activity.[29] To explore if product yields could be improved by increasing catalyst loadings, parallel assays were conducted with 0.5, 1.5, 5, 10, 20, 40, 60, and 100 mol% of **1**. Whereas a considerable yield improvement was observed when the mol% of catalyst was changed from 0.5 to 10 (Table 1, entries 25 *vs.* 24 *vs.* 23), the use of larger amounts of Fe catalyst did not result in improved yields (Table 1, entries 19 and 20 *vs.* 11 and 10 *vs.* 9 and 8 *vs.* 7 and 6). It should be outlined that, although modest, the TONs (5.8) obtained upon use of 0.5 mol% of **1** (Table 1, entry 25) substantiate the catalytic capacity of the system. However, nearly stoichiometric amounts of the product were obtained with 10 mol% of **1**, clearly showing a non-catalytic behaviour of the system under these conditions, which is consistent with the yield improvement observed with increasing reaction times. Given that our ultimate goal was the development of a synthetic method for the preparation of significant amounts of 8-OH-EFV, optimization led to the selection of conditions providing the best yield. These consisted of the use of an AcCN–AcOEt (1 : 1) solution of EFV, 10 mol% of Fe^{II}(bpmen)$(OTf)_2$, and hydrogen peroxide (1.35 eq.) in the presence of acetic acid (5.0 eq.) at 40 °C for 3 days, that resulted in a 12.0% yield of 8-OH-EFV (calculated from the HPLC peak areas). A preparative assay was performed, starting from 100 mg of EFV under the optimized conditions, resulting in 9.5% isolated yield of 8-OH-EFV (*cf.* Experimental section). Parallel reactions were performed to test different chromatographic purifications of the reaction mixture and, regardless of the technique used (*e.g.*, semi-preparative HPLC, column chromatography or preparative TLC) both the product and the starting material were isolated quantitatively. A scale-up study, starting from 1000 mg of EFV, revealed that the best results were

Table 1 Optimization of experimental conditions for the biomimetic oxidation of EFV into 8-OH-EFV

Entry	CAT[a]	CAT (%mol)	Time (h)	co-CAT	H_2O_2 (eq.)	AcCN–AcOEt	Yield[b] (%)
1	(**1**)	10		AcOH (0.5 eq.)	0.45	100 : 0	2.5
2		10	72	AcOH (5 eq.)	0.45	100 : 0	4.7
3		10	72		0.45	75 : 25	6.6
4		10	72		0.9	100 : 0	6.9
5		10	72		1.35[c]	100 : 0	5.7
6		100	72		1.35	50 : 50	2.1
7		100	2		1.35	50 : 50	1.0
8		60	24		1.35	50 : 50	1.3
9		60	2		1.35	50 : 50	1.1
10		20	24		1.35	50 : 50	2.2
11		20	2		1.35	50 : 50	1.3
12		10	24		1.35	30 : 80	0.6
13		10	2		1.35	30 : 80	0.5
14		10	2		1.35	40 : 60	0.9
15		10	24		1.35	40 : 60	0.9
16		10	24		1.35	50 : 50	1.3[d]
17		10	2		1.35	50 : 50	0.6[d]
18		10	5 min		1.35	50 : 50	1.8
19		10	2		1.35	50 : 50	3.2 (2.8)[e]
20		10	24		1.35	50 : 50	4.4 (5.0)[e]
21		10	48		1.35	50 : 50	4.8 (5.7)[e]
22		**10**	**72**		**1.35**	**50 : 50**	**12.0 (9.5)[e]**
23		10	72		1.35	100 : 0	7.7
24		1.5	72		1.35	100 : 0	4.4
25		0.5	72		1.35	100 : 0	2.9
26		10	72		[f]	100 : 0	3.7
27		10	72	None	0.45	100 : 0	<1
28	(**2**)	10	72	AcOH (5 eq.)	0.45	100 : 0	6.2
29	(**3**)	10	72		0.45	100 : 0	<1
30	(**4**)	10	72		0.45	100 : 0	<1
31	$Fe(OTf)_2$	10	72		0.45	100 : 0	n.r.[g]
32	None	None	72		0.45	100 : 0	n.r.
33[h]							4.8

[a] (**1**): $[Fe^{II}(bpmen)(OTf)_2]$;[29,30] (**2**): $[Fe(S,S\text{-PDP})(NCCH_3)_2]$ $(SbF_6)_2$;[31] (**3**): $[FeCl_2(Py_2S_2)]$;[32] (**4**): $[Cu(bpmen)(OTf)_2]$. [b] Unless specified, the reactions were conducted at 40 °C. The yields were calculated from the HPLC peak areas, corrected for the corresponding molar absorptivity at the monitored wavelength (254 nm). The numbers in parentheses represent the yields of isolated products. [c] Four sequential additions of 0.33 eq. of the oxidant at 1 hour intervals. [d] Reaction at room temperature. [e] Starting with 100 mg of EFV, the crude reaction mixture was purified by flash chromatography on silica (CH_2Cl_2 to 95 : 5 CH_2Cl_2–MeOH). [f] With 0.45 eq. of *m*-CPBA. [g] n.r., no reaction. [h] Fenton's oxidation conditions: $FeCl_2{\cdot}4H_2O$, 0.1 M HCl, 0.1 M H_2O_2.



Scheme 2 Direct hydroxylation of EFV mediated by the N_4-tetradentate Fe^{II} complex $[Fe(bpmen)(OTf)_2]$.

obtained at a 50 mM substrate concentration (*cf.* Experimental section).

In summary, direct EFV hydroxylation, using the $[Fe^{II}(bpmen)(OTf)_2]$–H_2O_2 oxidant system in the presence of acetic acid afforded the enantiomerically pure metabolite, 8-OH-EFV, in 9.5% isolated yield with nearly quantitative recovery of the unreacted EFV (*ca.* 98%). A three-cycle run provided a 22% total yield of the same metabolite (Scheme 2, *cf.* Experimental section). This cost-efficient one-pot strategy represents a considerable improvement when compared with the already available methods for the C8 hydroxylation of EFV,[12,13] avoiding a laborious multi-step synthetic approach and the resolution of racemic mixtures.

Contrasting with previously reported arene oxidations[29–36] catalyzed by Fe^{II} complexes, that involve hydroxylations of a series of simple aromatic substrates, our observation refers to an

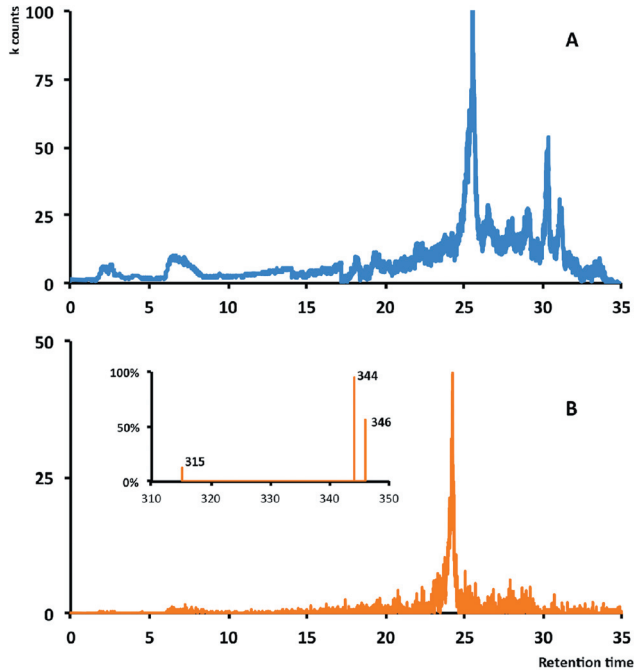


Fig. 1 (A) Total ion LC-ESI(−)-MS chromatogram of the reaction mixture obtained upon oxidation of 8-OH-EFV under the optimized conditions described in the text; (B) extracted ion chromatogram (*m*/*z* 344) and corresponding mass spectrum. The elution conditions are outlined in the Experimental section.

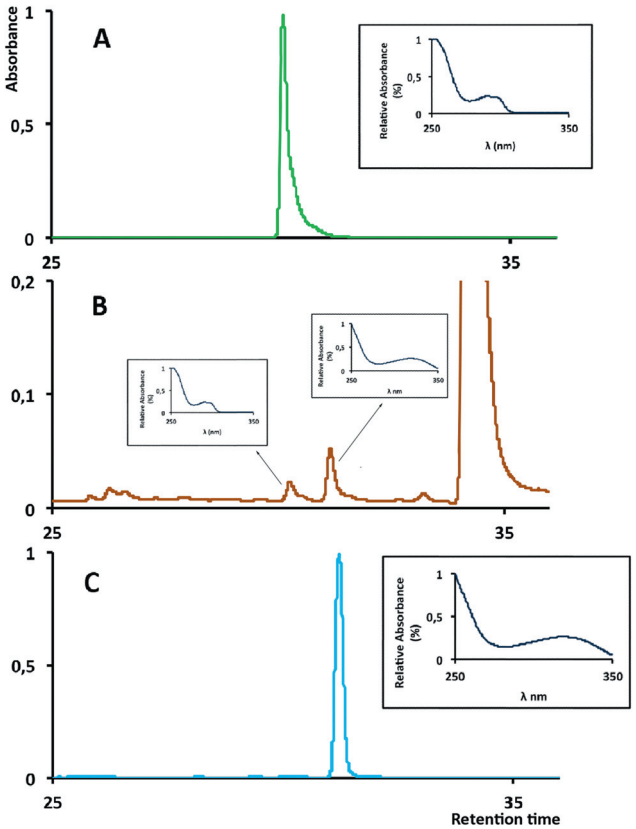
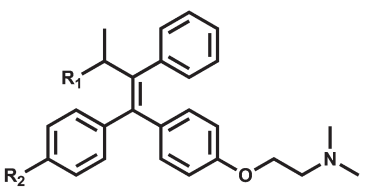


Fig. 2 HPLC-DAD analyses of: (A) standard of 7-OH-EFV; (B) the reaction mixture obtained upon EFV oxidation mediated by [Fe^{II}(bpmen)$(OTf)_2$] using 150 mM EFV and 1.8 eq. of H_2O_2; (C) standard of 8-OH-EFV. The elution conditions are outlined in the Experimental section. The insets represent the UV spectra obtained under the conditions of the HPLC run.

unprecedented complex molecule that is selectively converted into its major phenolic metabolite. Furthermore, the examples of most efficient catalysis mediated by nonheme Fe^{II} systems entail the *ortho*-hydroxylation of benzoic acids,[16,37,38] due to the favourable formation of a cyclic iron–arene complex involving the carbonyl group of the substrate. By contrast, the lack of an *ortho*-substituent capable of assisting catalysis typically results in a dramatic decrease of the hydroxylation efficiency. As such, the single-cycle 9.5% isolated yield obtained for EFV hydroxylation represents a very good conversion for a complex, rigid, and hindered core lacking catalytic-assisting groups. In fact, the EFV molecule displays an expected steric hindrance in the vicinity of the ring fusion, that namely inhibited the activity of bulky organic peroxides, such as *t*BuOOH, as discussed above. It should also be noted that, similarly to what is reported for the oxidation of other aromatic C–H bonds,[39–42] any attempts to improve the process efficiency led to partial degradation of the phenolic product, most likely due to further oxidation to catechols, and/or quinone species.[28,29] Indeed, LC-ESI(−)-MS/MS (*m*/*z* 344) analysis of the reaction mixture (Fig. 1) provided preliminary evidence for the formation of a quinone derivative upon oxidation of the isolated 8-OH-EFV under the optimized conditions described above.

Interestingly, when more concentrated solutions (>100 mM) were used, the formation of some byproducts of over-oxidation became significant. Indeed, upon the use of a larger excess of hydrogen peroxide (1.8 eq.) and a more concentrated EFV solution (150 mM), HPLC-DAD analysis provided evidence for the formation of the two phenolic EFV metabolites, 8-OH-EFV and 7-OH-EFV, in a ratio similar to that obtained under CYP

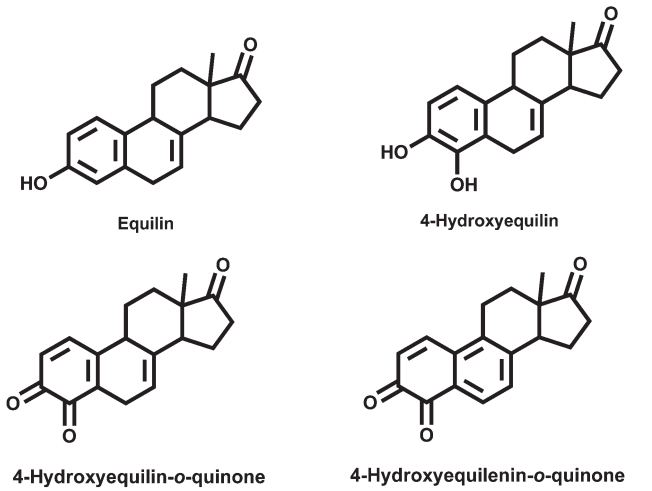


R_1=R_2=H;**Tamoxifen**
R_1=H, R_2=OH; **4-Hydroxytamoxifen**
R_1=OH, R_2=H; **α-Hydroxytamoxifen**

Scheme 3 Structures of tamoxifen and its hydroxylated metabolites, 4-hydroxytamoxifen and α-hydroxytamoxifen.

catalysis (Fig. 2).[43] The identification of the minor metabolite, 7-OH-EFV, was performed on the basis of undistinguishable UV profiles and identical retention times when compared with the corresponding synthetic standard under the same elution conditions.

To explore if this oxidation system could be successfully used to yield phenolic metabolites from other xenobiotics of interest, we oxidized the non-steroidal anti-estrogen tamoxifen (Scheme 3), extensively prescribed as an adjunct



Scheme 4 Structures of equilin and its metabolites 4-hydroxyequilin, 4-hydroxyequilin-*o*-quinone and 4-hydroxyequilenin-*o*-quinone.

chemotherapeutic agent against breast cancer,[44] and the equine estrogen equilin (Scheme 4), one of the components of Premarin, widely used in hormone replacement therapy.[45]

Tamoxifen undergoes multiple metabolic conversions, namely involving the formation of two hydroxylated metabolites, the phenol 4-hydroxytamoxifen and the allylic alcohol, α-hydroxytamoxifen (Scheme 3).[44] Upon oxidation of tamoxifen with the $[Fe^{II}(bpmen)(OTf)_2]$–H_2O_2 system (*cf.* Experimental section), the reaction mixture was analyzed by LC-ESI-MS/MS, which showed an ion of *m*/*z* 388, corresponding to the protonated mono-hydroxy-tamoxifen metabolites. Based upon comparison of retention times and MS/MS fragmentation patterns of the reaction mixture (Fig. 3, panel A) with those of the synthetic standards of 4-hydroxytamoxifen (Fig. 3, panel B) and α-hydroxytamoxifen (Fig. 3, panel C) we obtained unequivocal evidence for the formation of the phenolic tamoxifen metabolite.

Equilin is primarily metabolized *in vivo* to the catechol 4-hydroxyequilin, which is further oxidized to 4-hydroxyequilin-*o*-quinone and, ultimately, 4-hydroxyequilenin-*o*-quinone (Scheme 4).[45] LC-ESI-MS analysis of the reaction mixture obtained upon oxidation of equilin with the system described herein (*cf.* Experimental section) provided evidence for the presence of the protonated molecules of the catechol, 4-hydroxyequilin (*m*/*z* 285), and the quinones, 4-hydroxyequilin-*o*-quinone (*m*/*z* 283) and 4-hydroxyequilenin-*o*-quinone (*m*/*z* 281) (Fig. 4). These results demonstrate that the $[Fe^{II}(bpmen)(OTf)_2]$–H_2O_2 system can be used to access phenolic metabolites and their oxidized derivatives *in vitro*, in a biomimetic fashion that mirrors CYP activity.

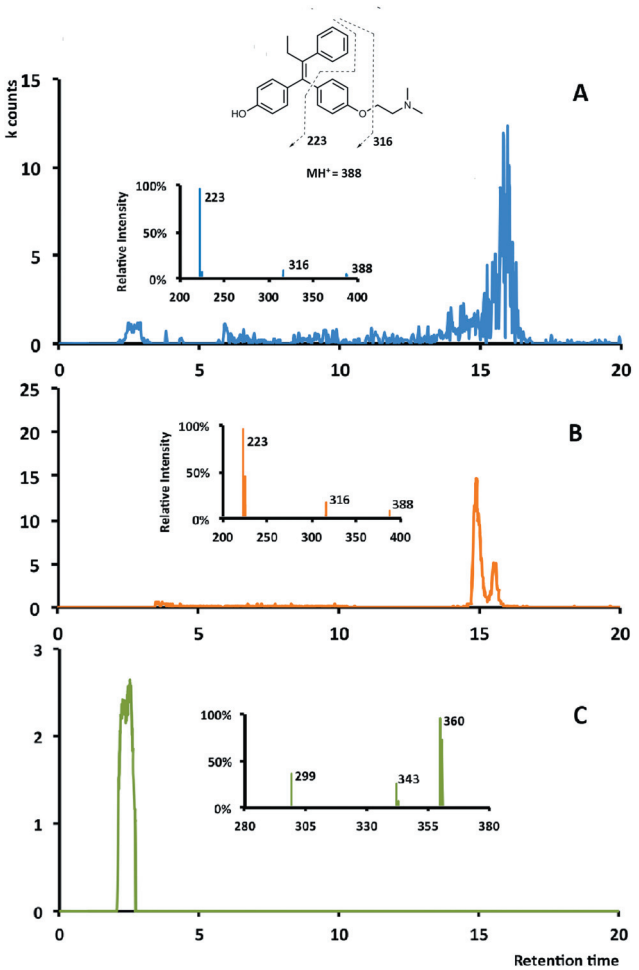


Fig. 3 LC-ESI-MS/MS (*m*/*z* 388) chromatogram and MS/MS mass spectra of: (A) reaction mixture obtained from oxidation of tamoxifen catalyzed by $[Fe^{II}(bpmen)(OTf)_2]$; (B) the synthetic standard of 4-hydroxytamoxifen (mixture of *E* and *Z* isomers); (C) the synthetic standard of α-hydroxytamoxifen. The elution conditions are outlined in the Experimental section.

Conclusions

We report herein the application of a flexible $[Fe^{II}(bpmen)(OTf)_2]$–H_2O_2 system to the oxidation of the first-line NNRTI EFV, which represents the first model for biomimetic oxidation of EFV. This oxidation system was successfully tuned for selectively providing the enantiomerically pure major EFV metabolite, 8-OH-EFV, in a single synthetic step. This EFV metabolite, obtained in a preparative scale, is now accessible in adequate amounts for molecular toxicology studies that are expected to clarify its relevance to the toxic events elicited by the parent drug. Additionally, upon tuning the experimental conditions, this system successfully simulated CYP-mediated EFV metabolism by providing the other reported phenolic EFV metabolite, 7-OH-EFV. Moreover, the successful oxidation of tamoxifen and equilin into their phenolic metabolites supports the general application of bio-inspired nonheme Fe-complexes for *in vitro* access to phenolic species (and their oxidized quinone derivatives), typically formed *via* CYP-mediated hydroxylation of aromatic compounds. The possibility of applying an oxidation system mirroring the CYP metabolic pathways anticipates the usefulness of the approach described herein for molecular toxicology studies of a large variety of toxic aromatic xenobiotics. Indeed, the present work represents a proof of concept regarding bioinspired oxidation technologies and opens new opportunities for this chemistry.

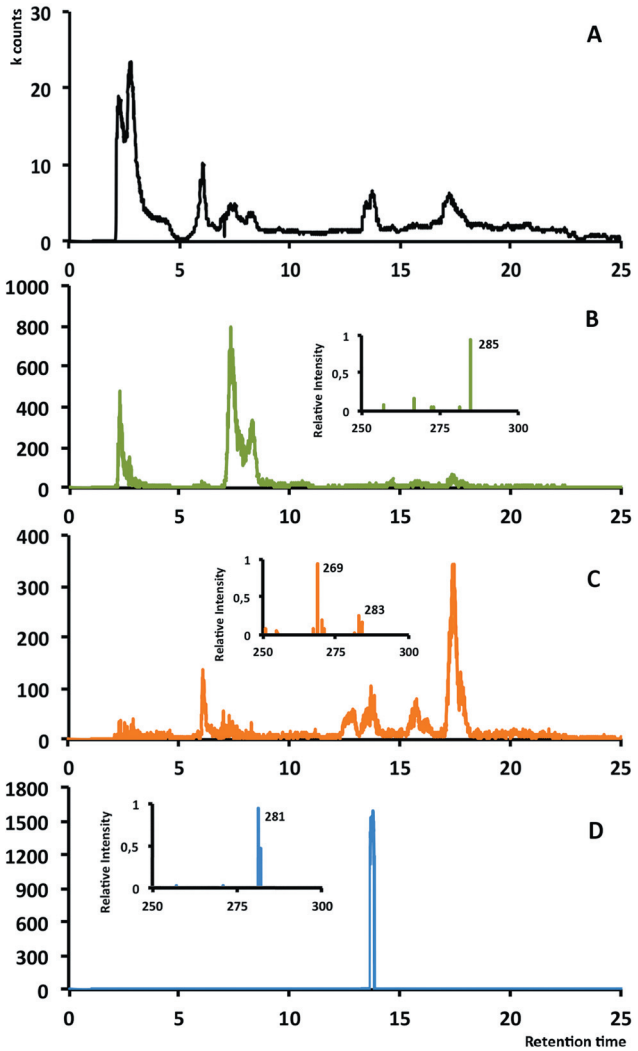


Fig. 4 (A) Total ion LC-ESI-MS chromatogram of the reaction mixture obtained upon oxidation of equilin mediated by $[Fe^{II}(bpmen)(OTf)_2]$. Extracted ion chromatograms and MS spectra of: (B) (*m*/*z* 285), corresponding to the protonated molecule of 4-hydroxyequilin; (C) (*m*/*z* 283), corresponding to the protonated molecule of 4-hydroxyequilin-*o*-quinone; and (D) (*m*/*z* 281) corresponding to the protonated molecule of 4-hydroxyequilenin-*o*-quinone. The elution conditions are outlined in the Experimental section.

Experimental

General remarks. Chemicals

Efavirenz was a gift from Dr F. A. Beland, National Center for Toxicological Research, Jefferson, Arkansas, USA. 7-OH-EFV[12] and the tamoxifen metabolites, α-hydroxytamoxifen and 4-hydroxytamoxifen (mixture of *E* and *Z* isomers),[44] were synthesized as described. All other commercially available reagents were acquired from Sigma-Aldrich Quimica, S.A. (Madrid, Spain), unless specified otherwise, and were used as received. Whenever necessary, solvents were purified by standard methods.[46]

Instrumentation

Melting temperatures were measured in a Leica Galen III hot stage apparatus and are uncorrected. The UV measurements were recorded on a Perkin Elmer Lambda 35 UV/VIS spectrophotometer. $[\alpha]_D$ Measurements were carried out in an LDT Automatic Polarimeter. HPLC was conducted on an Ultimate 3000 Dionex system consisting of an LPG-3400A quaternary gradient pump and a diode array spectrophotometric detector (Dionex Co., Sunnyvale, CA) and equipped with a Rheodyne model 8125 injector (Rheodyne, Rohnert Park, CA). HPLC analyses were performed with a Luna C18 (2) column (250 mm × 4.6 mm; 5 μm; Phenomenex, Torrance, CA), at a flow rate of 1 mL min^{-1}. Semipreparative HPLC separations were conducted with a Luna C18 (2) column (250 mm × 10 mm; 5 μm; Phenomenex) at a flow rate of 3 mL min^{-1}. A 30-min linear gradient from 5 to 70% acetonitrile in 0.1% aqueous formic acid, followed by a 2-min linear gradient to 100% acetonitrile and an 8-min isocratic elution with acetonitrile, was used in all instances. The UV absorbance was monitored at 254 nm. Mass spectra (MS) were recorded on a 500-MS LC ion trap mass spectrometer (Varian, Inc., Palo Alto, CA), operated in the positive and negative electrospray ionization (ESI) modes. The spray voltage was set at ±5 kV and the capillary voltage was set at 10 V. LC-ESI-MS/MS analyses were performed with the same spectrometer, coupled to a Varian ProStar 410 autosampler, two 210-LC chromatography pumps, and a ProStar 335 diode array detector. Data acquisition and processing were performed using Varian MS Control 6.9 software. The samples were injected onto the column *via* a Rheodyne injector with a 20 μL loop. Separations were conducted at 30 °C, using a Luna C18 (2) column (150 mm × 2 mm, 3 μm; Phenomenex). The mobile phase was delivered at a flow rate of 200 μL min^{-1}, using a 5-min isocratic elution with 5% acetonitrile in 0.1% aqueous formic acid, followed by a 30 min linear gradient from 5 to 70% acetonitrile, a 2 min linear gradient to 100% acetonitrile, and an 8 min isocratic elution with acetonitrile. The mass spectrometer was operated in the positive and negative ESI mode; the optimized operating parameters were: ion spray voltage, +5.2 kV; capillary voltage, 20 V; and RF loading, 90%. Nitrogen was used as the nebulizing and drying gas, at pressures of 50 and 30 psi, respectively; the drying gas temperature was 350 °C. MS/MS spectra were obtained with an isolation window of 1.5 Da, excitation energy values between 0.9 and 1.2 V, and an excitation time of 10 ms.

^1H NMR spectra were recorded on Bruker Avance III 400 or 500 spectrometers (Bruker BioSpin GmbH, Rheinstetten, Germany) operating at 400 and 500 MHz, respectively. ^{13}C NMR spectra were recorded on the same instruments, operating at 100.62 and 125.77 MHz, respectively. Chemical shifts are reported in ppm downfield from tetramethylsilane, and coupling constants (*J*) are reported in Hz. Resonance and structural assignments were based on the analysis of coupling patterns, including the ^{13}C–^1H coupling profiles obtained in bidimensional heteronuclear multiple bond correlation (HMBC) and heteronuclear single quantum coherence (HSQC) experiments, performed with standard pulse programs.

Oxidations of efavirenz

General method for optimization of experimental conditions. Acetic acid (9 μL) was added to a solution of EFV (10 mg, 32 μmol) and catalyst (0–100% mol) in a suitable solvent (500 μL) (Table 1). H_2O_2 (35 wt%, 0.45, 0.9, or 1.35 eq.) was

added at r.t. The final mixtures were stirred either at r.t. or at 40 °C. For monitorization purposes aliquots (50 μL) were collected after 5 min, 2, 24, 48, and 72 h. Sodium bisulfite (100 μL, sat. aq. solution) was added and the resulting solution was extracted with AcOEt (50 μL) and monitored by HPLC-DAD at 254 nm.

Optimized conditions

Acetic acid (91 μL, 1.6 mmol, 5 eq.) was added to a solution of EFV (100 mg, 320 μmol) and catalyst (10% mmol) in AcCN–AcOEt (5 mL, 1 : 1). The solution was stirred and H_2O_2 (35 wt %, 37 μL, 430 μmol, 1.35 eq.) was added at r.t. The final mixture was stirred at 40 °C for 3 days and the reaction progress was monitored by HPLC. The mixture was then treated with sodium bisulfite (1 mL, sat. aq. solution) and AcOEt (2 mL) and the organic fraction was washed sequentially with aqueous $NaHCO_3$, water and brine, dried over anhydrous Na_2SO_4, and evaporated. The crude mixture was purified by flash chromatography on silica (CH_2Cl_2 to 95 : 5 CH_2Cl_2–MeOH), yielding 8-OH-EFV (10 mg, 9.5%). The unreacted EFV was recovered quantitatively (87 mg).

(*S*)-6-Chloro-4-(cyclopropylethynyl)-8-hydroxy-4-(trifluoromethyl)-1,4-dihydro-2*H*-3,1-benzoxazin-2-one (8-OH-EFV). M_p 171–172 °C (lit[13] 170–172 °C); MS [ESI(+)]: *m/z*: 354 [(^{35}Cl)M + Na]$^+$; 332 [(^{35}Cl)M + H]$^+$; MS [ESI(−)]: *m/z*: 330 [(^{35}Cl)M − H]$^-$, 286 [(^{35}Cl)M − (CO_2)]$^-$, 258 [M − (CO_2) − (C_2H_4)]$^-$; $[\alpha]_D$ (25 °C) = −30°, measured at 230 mg per 100 mL in methanol (lit: −29,3°,[12] and −32.0°,[13]); ^{1}H-NMR (CD_3OD): 6.93 (d, 1H, *J* = 2.15 Hz, H7), 6.92 (d, 1H, *J* = 2.15 Hz, H5), 1.48 (m, 1H, cyclopropyl-CH), 0.97–0.92 (m, 2H, cyclopropyl-CH_2), 0.82–0.77 (m, 2H, cyclopropyl-CH_2); ^{1}H-NMR (DMSO-d_6): 11.03 (br s, 1H, NH/OH), 10.3 (br s, 1H, NH/OH), 6.94 (d, 1H, *J* = 2.19 Hz, H7), 6.88 (d, 1H, *J* = 2.19 Hz, H5), 1.58 (m, 1H, cyclopropyl-CH), 0.87–0.84 (m, 2H, cyclopropyl-CH_2), 0.79–0.74 (m, 2H, cyclopropyl-CH_2); ^{13}C-NMR (CD_3OD): 149.4 (C═O), 146.4 (C8), 129.6 (C6), 129.2 (C10), 118.4 (C7), 117.8 (C5), 116.6 (C9), 96.6 (C13), 76.8 (q, C(4), J^2 = 35 Hz), 67.6 (C12), 9.09 (C15/C16), 9.06 (C15/C16), −0.2 (C14); ^{13}C-NMR (DMSO-d_6): 146.7 (C═O), 145.9 (C8), 127.7 (C6), 124.2 (C10), 117.3 (C7), 116.7 (C5), 115.2 (C9), 95.8 (C13), 77.6 (q, C(4), J^2 = 35 Hz), 66.5 (C12), 8.99 (C15/C16), 8.95 (C15/C16), −0.81 (C14).

Three-cycles run. The EFV obtained from the reaction described above (87 mg, 270 μmol) was dissolved in AcCN–AcOEt (4.4 mL, 1 : 1). Following the addition of catalyst (10% mol) acetic acid (79 μL, 1.39 mmol, 5 eq.) was added. The solution was stirred and H_2O_2 (35 wt%, 31 μL, 360 μmol, 1.35 eq.) was added at r.t. The final mixture was stirred at 40 °C for 3 days. The mixture was then treated with sodium bisulfite (1 mL, sat. aq.) and AcOEt (2 mL) and the organic fraction was washed sequentially with aqueous $NaHCO_3$, water and brine, dried over anhydrous Na_2SO_4, and evaporated. The crude mixture was purified by flash chromatography on silica (CH_2Cl_2 to 95 : 5 CH_2Cl_2–MeOH), yielding 8-OH-EFV (8 mg, 9%). The recovered EFV (74 mg, 230 μmol), was reacted again under the described conditions affording 8-OH-EFV (7 mg, 9%). The overall yield of 8-OH-EFV was 21.8% (23 mg) after the three-cycles run.

Scale up reaction. Acetic acid (0.9 mL, 16 mmol, 5 eq.) was added to a solution of EFV (1 g, 3.2 mmol) and catalyst (10% mol) in AcCN–AcOEt (64 mL, 1 : 1). The solution was stirred and H_2O_2 (35 wt%, 380 μL, 4.3 mmol, 1.35 eq.) was added dropwise at r.t. The final mixture was stirred at 40 °C for 3 days and the reaction progress was monitored by HPLC. The mixture was then treated with sodium bisulfite (10 mL, sat. aq. solution) and AcOEt (20 mL) and the organic fraction was washed sequentially with aqueous $NaHCO_3$, water and brine, dried over anhydrous Na_2SO_4, and evaporated. The crude mixture was purified by flash chromatography on silica (CH_2Cl_2 to 95 : 5 CH_2Cl_2–MeOH), yielding 8-OH-EFV (65 mg, 6.2%).

Oxidation of 8-OH-EFV. Acetic acid (4.3 μL, 76 μmol, 5 eq.) was added to a solution of 8-OH-EFV (5 mg, 15 μmol) and catalyst (10% mol) in AcCN–AcOEt (250 μL, 1 : 1). H_2O_2 (35 wt%, 1.8 μL, 20 μmol, 1.35 eq.) was added at r.t. The final mixture was stirred at 40 °C for 24 h and the reaction progress was monitored by HPLC-DAD and HPLC-ESI-MS.

Oxidation of equilin. Acetic acid (10 μL, 187 μmol, 5 eq.) was added to a solution of equilin (10 mg, 37 μmol) and catalyst (10% mol) in AcCN–AcOEt (500 μL, 1 : 1). Following addition of H_2O_2 (35 wt%, 4.4 μL, 50 μmol, 1.35 eq.) at r.t. the final mixture was stirred at 40 °C for 24 h. The reaction mixture was monitored by HPLC-DAD and LC-ESI-MS after 1 and 24 h.

Oxidation of tamoxifen. Acetic acid (8 μL, 135 μmol, 5 eq.) was added to a solution of tamoxifen (10 mg, 27 μmol) and catalyst (10% mol) in AcCN–AcOEt (500 μL, 1 : 1). Following addition of H_2O_2 (35 wt%, 3.7 μL, 36 μmol, 1.35 eq.) at r.t. the final mixture was stirred at 40 °C for 24 h. The reaction mixture was monitored by HPLC-DAD and LC-ESI-MS/MS after 1 and 24 h.

Abbreviations

Bpmen	*N*,*N*′-bis-(2-pyridylmethyl)-1,2-diaminoethane
CYP	cytochrome P450
ESI	electrospray ionization
EFV	efavirenz
HIV	human immunodeficiency virus type 1
HPLC-DAD	high performance liquid chromatography with diode array detection
MS/MS	tandem mass spectrometry
NNRTI	non-nucleoside reverse transcriptase inhibitor
S,*S*-PDP	2-({(*S*)-2-[(*S*)-1-(pyridin-2-ylmethyl)pyrrolidin-2-yl]pyrrolidin-1-yl}methyl)pyridine
TLC	thin layer chromatography
Py_2S_2	1,6-bis(2′-pyridyl)-2,5-dithiahexane

Acknowledgements

We thank the Portuguese NMR and MS Networks (IST-UTL Nodes) for providing access to the facilities. This work was supported in part by Fundação para a Ciência e a Tecnologia (FCT), Portugal, through research grant PTDC/QUI-QUI/113910/2009

and pluriannual funds to Centro de Química Estrutural (PEst-OE/QUI/UI0100/2011). RW and SGH also thank FCT for postdoctoral (SFRH/BPD/70953/2010) and doctoral (SFRH/BD/80690/2011) fellowships, respectively.

References

1 M. A. Thompson, J. A. Aberg, P. Cahn, J. S. G. Montaner, G. Rizzardini, A. Telenti, J. M. Gatell, H. F. Günthard, S. M. Hammer, M. S. Hirsch, D. M. Jacobsen, P. Reiss, D. D. Richman, P. A. Volberding, P. Yeni and R. T. Schooley, *JAMA*, 2010, **304**, 321.
2 P. Lochet, H. Peyrière, A. Lotthé, J. M. Mauboussin, B. Delmas and J. Reynes, *HIV Med.*, 2003, **4**, 62.
3 G. I. T. Cavalcante, V. L. M. Capistrano, F. S. D. Cavalcante, S. M. M. Vasconcelos, D. S. Macêdo, F. C. F. Sousa, D. J. Woods and M. M. F. Fonteles, *Int. J. Neurosci.*, 2010, **120**, 739.
4 U. Mehta and G. Maartens, *Lancet Infect. Dis.*, 2007, **7**, 733.
5 A. E. Mutlib, R. J. Gerson, P. C. Meunier, P. J. Haley, H. Chen, L. S. Gan, M. H. Davies, B. Gemzik, D. D. Christ, D. F. Krahn, J. A. Markwalder, S. P. Seitz, R. T. Robertson and G. T. Miwa, *Toxicol. Appl. Pharmacol.*, 2000, **169**, 102.
6 A. E. Mutlib, H. Chen, G. A. Nemeth, J. A. Markwalder, S. P. Seitz, L. S. Gan and D. D. Christ, *Drug Metab. Dispos.*, 1999, **27**, 1319.
7 N. N. Bumpus, *Toxicol. Appl. Pharmacol.*, 2011, **257**, 227.
8 E. Ngaimisi, S. Mugusi, O. M. Minzi, P. Sasi, K.-D. Riedel, A. Suda, N. Ueda, M. Janabi, F. Mugusi, W. E. Haefeli, J. Burhenne and E. Aklillu, *Clin. Pharmacol. Ther.*, 2010, **88**, 676.
9 J. S. Barrett, A. S. Joshi, M. Chai, T. M. Ludden, W. D. Fiske and H. J. Pieniaszek, *Int. J. Clin. Pharmacol. Ther.*, 2002, **40**, 507.
10 S. R. Faucette, T.-C. Zhang, R. Moore, T. Sueyoshi, C. J. Omiecinski, E. L. LeCluyse, M. Negishi and H. Wang, *J. Pharmacol. Exp. Ther.*, 2007, **320**, 72.
11 S. Mouly, K. S. Lown, D. Kornhauser, J. L. Joseph, W. D. Fiske, I. H. Benedek and P. B. Watkins, *Clin. Pharmacol. Ther.*, 2002, **72**, 1.
12 J. A. Markwalder, D. D. Christ, A. Mutlib, B. C. Cordova, R. M. Klabe and S. P. Seitz, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 619.
13 D. D. Christ, A. J. Cocuzza, S. S. Ko, J. A. Markwalder, A. E. Mutlib, R. L. Parsons, Jr, M. Patel and S. P. Seitz, U.S. Pat. 5,874,430, 1999.
14 L. Que Jr, in *Bioinorganic Catalysis*, ed. J. Reedjik and E. Bouwman, Marcel Dekker Inc., New York, USA, 1999, (ch.10).
15 K. Chen, M. Costas, J. Kim, A. K. Tipton and L. Que, Jr, *J. Am. Chem. Soc.*, 2002, **124**, 3026.
16 S. Taktak, M. Flook, B. M. Foxman, L. Que, Jr and E. V. Rybak-Akimova, *Chem. Commun.*, 2005, 5301.
17 V. Balland, D. Mathieu, N. Pons-Y-Moll, J. F. Bartoli, F. Banse, P. Battioni, J.-J. Girerd and D. Mansuy, *J. Mol. Catal. A: Chem.*, 2004, **215**, 81.
18 M. Costas, K. Chen and L. Que, Jr, *Coord. Chem. Rev.*, 2000, **200**, 517.
19 K. Chen and L. Que, Jr, *Chem. Commun.*, 1999, 1375.
20 D. Quiñonero, K. Morokuma, D. G. Musaev, R. Mas-Ballesté and L. Que, Jr, *J. Am. Chem. Soc.*, 2005, **127**, 6548.
21 B. Testa and S. D. Krämer, *The Biochemistry of Drug Metabolism: Principles, Redox Reactions, Hydrolyses. Part 2. Redox Reactions and their Enzymes*, Wiley-VCH, Weinheim, Germany, 2008.
22 J. Bernadou and B. Meunier, *Chem. Commun.*, 1998, 2167.
23 M. S. Chen and M. C. White, *Science*, 2007, **318**, 783.
24 R. R. Fernandes, J. Lasri, M. F. C. Guedes da Silva, J. A. L. da Silva, J. J. R. Fraústo da Silva and A. J. L. Pombeiro, *Appl. Catal., A: General*, 2011, **402**, 110.
25 G. J. P. Britovsek, J. England and A. J. P. White, *Inorg. Chem.*, 2005, **44**, 8125.
26 S. Poussereau, G. Blondin, M. Cesario, J. Guilhem, G. Chottard, F. Gonnet and J.-J. Girerd, *Inorg. Chem.*, 1998, **37**, 3127.
27 N. A. Vermeulen, M. S. Chen and M. C. White, *Tetrahedron*, 2009, **65**, 3078.
28 M. Costas, M. P. Mehn, M. P. Jensen and L. Que, Jr, *Chem. Rev.*, 2004, **104**, 939.
29 O. V. Makhlynets and E. V. Rybak-Akimova, *Chem.–Eur. J.*, 2010, **16**, 13995.
30 J. P. Hage and D. T. Sawyer, *J. Am. Chem. Soc.*, 1995, **117**, 5617.
31 D. Mathieu, J. F. Bartoli, P. Battioni and D. Mansuy, *Tetrahedron*, 2004, **60**, 3855.
32 J.-F. Bartoli, F. Lambert, I. Morgenstern-Badarau, P. Battioni and D. Mansuy, *C. R. Chim.*, 2002, **5**, 263.
33 H. C. Tung, C. Kang and D. T. Sawyer, *J. Am. Chem. Soc.*, 1992, **114**, 3445.
34 D. Bianchi, M. Bertoli, R. Tassinari, M. Ricci and R. Vignola, *J. Mol. Catal. A: Chem.*, 2003, **204–205**, 419.
35 T. Tezuka, N. Narita, W. Ando and S. Oae, *J. Am. Chem. Soc.*, 1981, **103**, 3045.
36 S. Udenfriend, C. T. Clark, J. Axelrod and B. B. Brodie, *J. Biol. Chem.*, 1954, **208**, 731.
37 S. P. de Visser, K. Oh, A.-R. Han and W. Nam, *Inorg. Chem.*, 2007, **46**, 4632.
38 N. Y. Oh, M. S. Seo, M. H. Lim, M. B. Consugar, M. J. Park, J.-U. Rohde, J. Han, K. M. Kim, J. Kim, L. Que, Jr and W. Nam, *Chem. Commun.*, 2005, 5644.
39 T. Kurata, Y. Watanabe, M. Katoh and Y. Sawaki, *J. Am. Chem. Soc.*, 1988, **110**, 7472.
40 D. T. Sawyer, A. Sobkowiak and T. Matsushita, *Acc. Chem. Res.*, 1996, **29**, 409.
41 D. A. Wink, R. W. Nims, J. E. Saavedra, W. E. Utermahlen, Jr and P. C. Ford, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 6604.
42 *Iron Catalysis: Fundamentals and Applications (Topics in Organometallic Chemistry)*, ed. B. Plietker, Springer-Verlag, Berlin, Germany, 2011, vol. 33.
43 B. A. Ward, J. C. Gorski, D. R. Jones, S. D. Hall, D. A. Flockhart and Z. Desta, *J. Pharmacol. Exp. Ther.*, 2003, **306**, 287.
44 G. Gamboa da Costa, L. P. McDaniel-Hamilton, R. H. Heflich, M. M. Marques and F. A. Beland, *Carcinogenesis*, 2001, **22**, 1307.
45 F. Zhang, Y. Chen, E. Pisha, L. Shen, Y. Xiong, R. B. van Breemen and J. L. Bolton, *Chem. Res. Toxicol.*, 1999, **12**, 204.
46 D. D. Perrin and W. L. F. Armarego, *Purification of Laboratory Chemicals*, Pergamon Press, Oxford, UK, 3rd edn, 1988, pp. 1–391.