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Fe(II)-Induced Reduction of Labelled Endoperoxides. NMR Degradation Studies on G3 Factor and Its Methyl Ether

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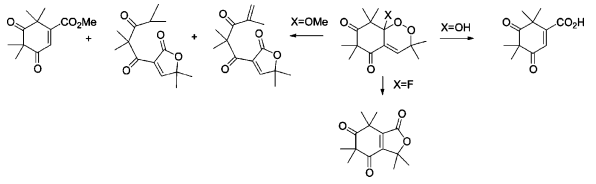

The behavior of G3 factor and of its methylated or fluorinated analogues G3Me and G3F, was studied under Fe(II) conditions. Degradation products were isolated and characterized in each case. The use of labelled compounds allowed us to propose mechanisms in which a tertiary radical is involved. This radical rearranges by *5-exo*-trig cyclization, or disproportionates in the case of G3Me. A correlation between antiplasmodial activity and stability of this radical is proposed.

Research for new therapeutic agents against malaria, one of the major infectious diseases, is still the subject of intensive developments because of the multiplication of multidrug-resistant *Plasmodium* strains. As suggested from biochemical studies, several efficient antiparasitic drug families interfere with the redox defense system of the parasite and the ways in which they act begin to be better understood. Among them, artemisinin and more generally peroxyketals such as BO7, arteflene, and other natural or synthetic peroxyketals[1−5] emerge in the fight against malaria.

As we were concerned by bicyclic endoperoxides,[6] which act as phytohormones and contribute to frost and hydric stress resistance in *Eucalyptus* and *Myrtacea* species, we studied the possible activity of these compounds against malaria. These phytohormones, also called G-factors, are easily extracted from *Eucalyptus grandis* and other myrtles potentially constituting a good base for modified analogues. They present a peroxyhemiketalic structure and are biosynthesized in the plant in response to stress.[7] The metabolic pathway involves an unknown masked precursor, probably derived from syncarpic acid, 2,2,4,4-tetramethyl-1,3,5-tricyclohexanone, a natural product also available by extraction or chemical synthesis.[7]

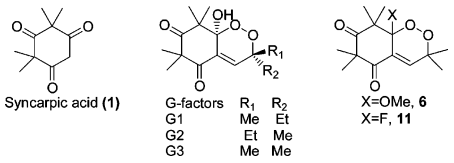

FIGURE 1. Syncarpic acid, G-factors and synthetic derivatives **6** and **11**.

Endoperoxides, active against malaria, are thought to interact with an iron-protoporphyrin of the heme coming from hemoglobin destruction of infected blood cells. The redox system was found to be irreversible for artemisinin, and the pathway[8] begins by an activation of the peroxo-bridge by free cellular iron(II) or heme iron. Then, a homolytic opening of the O−O bond occurs, the reduction of the peroxo-bridge giving rise to oxygenated radicals that rearrange themselves as carbon-centered radicals,[9] able to alkylate the heme.[10] Wu[11] described the mechanism of homolytic opening of the O−O bond in artemisinin. Meunier reported the complete characterization of the heme artemisinin adduct.[12−14] Recently, in vitro alkylation of human hemoglobin by artemisinin was described,[15] and the participation of carbon-centred radicals is nowadays commonly acknowledged. Recent results are also consistent with the alkylation of proteins, which can constitute other possible targets for artemisinin.[16,17] An alternative view proposed by Haynes and Krishna[18] consists of the generation of reactive oxygen species.

From our previous studies[19−21] on the G factor family, we were pleased to identify some compounds with good antiparasitic activities, even if they are not yet at the

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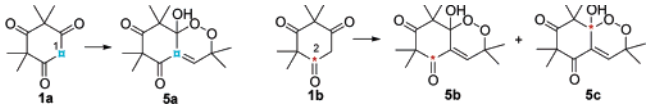
artemisinin level. Several parameters were found to be important for their antimalarial efficiency: lipophilicity, redox potential, and the nature of the function on the hemiketalic carbon, a point which seemed to be crucial. For example, endoperoxide G3 (**5**) completely lost antimalarial activity when fluorinated on hemiketal position (**11**) (IC_{50} > 100μM on Nigerian strains).[21] More intriguing was the difference observed when the hemiketalic OH was replaced by OMe. The biological activity, tested in vitro on *Plasmodium falciparum* infected cells, was increased by a factor of nearly 100 for the etherified endoperoxide (IC_{50}(**6**) = 0.28 μM and IC_{50}(**5**) = 36 μM on Nigerian strains).[20] The observation that a cyclic peroxyhemiketal is less antimalarially potent than its corresponding cyclic peroxyketal has been reported before.[22] To understand why, we first examined both compounds electrochemically, using cyclic voltammetry and thin layer voltammetry. One electron was detected during the reduction of both compounds and a slow and irreversible system was observed, in agreement with other experiments on endoperoxides.[20] A slight difference can be noticed between the relative peak potential values of compounds **5** and **6**. The standard potential was also determined for these two reference compounds according to Marcus theory. Whereas a concerted mechanism was observed for artemisinin,[23,24] competition between two electron-transfer mechanisms, a concerted one and a stepwise mechanism, was demonstrated for the first time for both **5** and for **6**.[25] Nevertheless this interesting result did not explain the differences encountered in the biological activity of the two compounds, as they present quite similar standard potentials as artemisinin and their O−O bond dissociation energies (BDE) are in the same range.

A chemical approach to the reduction process was then undertaken. We studied the Fe(II)-induced reduction of G3 (**5**), G3Me (**6**), and **11** and then characterized the degradation products. The conditions ($FeSO_4$/CH_3CN−H_2O, 1/1) were chosen to be closer to biological conditions than the THF-based system.[11] In that case pH medium is acidic and mimics the pH of the parasite food vacuole (pH 5.0−5.4). ^{13}C-labelled derivatives for compounds **5** and **6** were first synthesized to allow a better identification of the final products and to highlight the chemical rearrangements occurring. The results and the mechanisms we thought to be involved in the reduction process are presented here, and the antimalarial activity data will be tentatively correlated to these observations.

Syncarpic acid was labelled by using $^{13}C_1$- or $^{13}C_2$-labelled acetyl chloride in the synthesis. ^{13}C-1 syncarpic acid (**1a**) and ^{13}C-2 or ^{13}C-6 **1b** (due to the symmetry of the compound) were obtained from C-acetylation of ethyl-

SCHEME 1. Synthesis of Labelled Endoperoxides



2,2,4,4-tetramethyl β-ketoester, followed by intramolecular cyclization. The β-ketoester results from a condensation step between an isobutyl ester enolate and isobutyryl chloride.

^{13}C-Labelled endoperoxides were synthesized from syncarpic acid as previously described.[6] One equivalent of isobutyraldehyde is added to 1 equiv of piperidine to form iminium **2**, which is then added to syncarpic acid **1**, affording the Mannich base **3**, stable in aprotic medium. In acidic and protic medium, the enone **4**, in equilibrium with its dienol, is released and further transformed by autoxidation by dioxygen to yield endoperoxide **5**.

Endoperoxide **5a** is obtained from ^{13}C-1 syncarpic acid **1a**. Endoperoxide **5b** and **5c** were obtained from ^{13}C-2 or ^{13}C-6 syncarpic acid **1b**, in a 1/1 mixture, the synthesis inducing desymmetrization. Methylation of the hemiketalic position was then performed on compounds **5** and **5b/5c** using butyllithium labelled or unlabelled methyl triflate, to give the etherified compounds **6a** and **6b/6c**, respectively (Scheme 1).

Comparative reduction experiments were carried out on labelled and unlabelled **5** and **6** and on the unlabelled fluoroendoperoxide **11**, obtained by treating G3 compound with diethylaminosulfurtrifluoride as described earlier.[20] The reactions were carried out under argon in aqueous acetonitrile in the presence of 1 molar equiv of ferrous sulfate. The medium becomes acid as a result of the release of sulfuric acid (a drop in the pH of the reaction mixture is indeed noticed, and pH was found around 5). It is worth mentioning that 1 equiv of $FeSO_4$ is necessary for the reduction of **5**, **6**, and **11**. Catalytic amounts of $FeSO_4$ did not allow reduction, reactions simply not occurring at all. This observation was surprising since regeneration of Fe(II) was expected to occur in the reduction process. The endoperoxides probably first coordinate to Fe(II) and then form complexes with Fe(II). A few minutes after the beginning of the reaction the complexes issued from **5** or **6** could be isolated as white amorphous powder, by evaporating acetonitrile and freeze-drying water. Unfortunately, we did not succeed in crystallizing them for XR diffraction analysis. However, a cyclic voltammetry study showed a reversible wave at 0.65 V in oxidation, characterizing an Fe(II) complex. Such Fe(II) complexes were previously suggested by Haynes[26] for piperazine derivatives of artemisinin.

NMR analysis of intermediate reaction mixtures and chromatograms (12 h) are shown in supplementary data. It was quite surprising that endoperoxides **5** or **6** were still present, indicating a slow decomposition. As redox properties ($E°$) and BDE are close to that of artemisinin, the slowness of the reduction process could be explained by the formation of iron(II) complexes blocking the way

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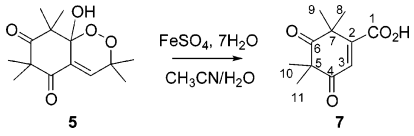
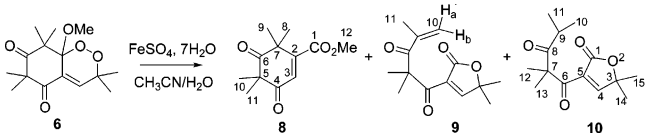
SCHEME 2. Reduction of Compound 5



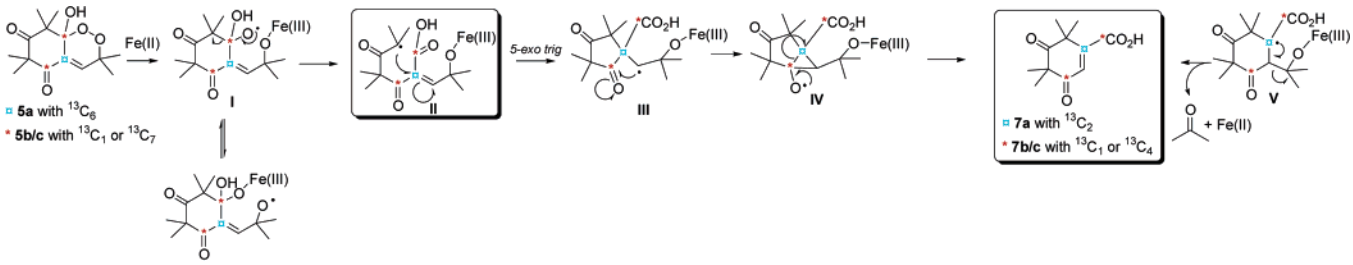
SCHEME 3. Reduction of Compound 6



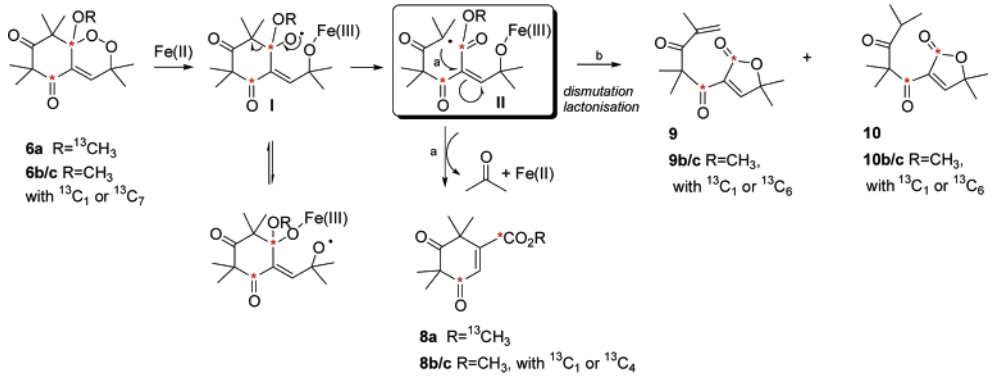
to Fe(II) and the necessity to use 1 equiv of $FeSO_4$ for the reaction to occur. Reactions with compounds **5** or **6** were stopped after 24 h and for **11** after 4 h; end products were then analyzed. A major product **7** (Scheme 2) was obtained in 82% yield from **5**, whereas in the same conditions, after careful purification, three major compounds, **8**, **9**, and **10** (respectively, 23%, 24%, and 24%), were isolated from **6** (Scheme 3). In both cases starting material was present (about 5%). Only one major compound **12** (Scheme 6) was generated by reduction of the fluoroendoperoxide **11**. Compounds **7**−**10** and **12** were mainly identified from NMR analysis, IR, and mass spectroscopy data and were obtained with >95% purity as estimated by ^{1}NMR spectroscopy and HPLC analysis.

Compound **7**, extracted first from experiments on unlabelled G3 and then from the labelled samples, was studied by NMR (^{1}H and ^{13}C spectra, HMBC), and a tetramethyl-di-oxo-cyclohexenyl acid structure agrees with the results. The ^{13}C NMR spectrum of compound **7a** shows a particularly intense peak that corresponds to ^{13}C-2 (158.6 ppm), a very surprising result since the labelled carbon was α to the carbonyl in G3 **5a** but in the *β* position in degradation product **7a**. In the ^{1}H NMR spectrum, a doublet can be attributed to C-8, C-9 methyl groups at 1.54 ppm, and the coupling constant (^3J = 5 Hz) is due to the ^3J coupling with C-2. The 1/1 mixture of compounds **7b/c**, each labelled at a different position, presents two intense ^{13}C peaks easily attributed to carbons ^{13}C-1 (169.9 ppm) and ^{13}C-4 (201.7 ppm). The ^{1}H spectrum is more complex since it results from the superimposition of two spectra for chemically identical but differently labelled compounds. Indeed, the methyl groups of C-10 and C-11 (1.34 ppm) are formed by the superimposition of two patterns: a doublet due to coupling constant ^3J with ^{13}C-4 (at 50% in the solution) and a singlet for both methyl groups, each being too far from ^{13}C-1 to allow coupling. The signal for H-3 (6.52 ppm) was expected to be a doublet of doublets, with respect to the two possible coupling constants ^2J (H-C-4) and ^3J (H-C-1), but it appeared as a doublet, indicating that one constant is probably equal to zero. This point was further confirmed by heteronuclear selective decoupling experiments for compounds **7b/c**. ^{13}C decoupling on C-4 was achieved by applying reduced power to its resonance frequency (201.7). It shows a simplification for the methyl group signals C-10 and C-11 but not for H-3. Thus, these methyl groups couple with the carbon C-4

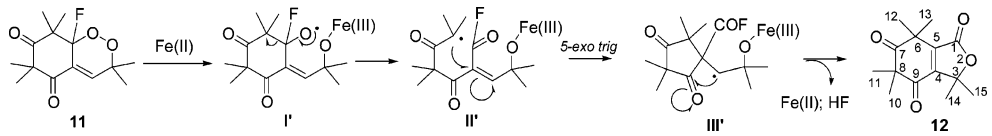
SCHEME 4. Conceivable Mechanism for 5a−c Reduction



SCHEME 5. Conceivable Mechanism for Reduction of Labelled 6



SCHEME 6. Reduction of Endoperoxide 11 and Conceivable Mechanism



and a long-range coupling constant ^3J (5 Hz) can be measured. H-3 is coupled with C-1 with a ^3J value of 10 Hz. The ^2J coupling constant between H-3 and C-4 was equal to zero, in agreement with the absence of a correlation spot on HMBC spectra. A C-1 decoupling experiment confirmed the previous result: the signal for H-3 is the only one simplified due to the suppression of ^3J coupling between H-3 and C-1.

Interestingly, Schobert and co-workers described synthesis and $FeBr_2$ reduction of stable endoperoxides. They obtained a butenolide as major compound, which is consistent with the formation as intermediate of a tertiary radical following the same rearrangement after homolytic opening of the O−O bond.[27]

Ester **8a** and lactones **9** and **10** were obtained from labelled precursor **6a**. The ^{13}C NMR spectrum of **8a** exhibits an intense peak at 53.0 ppm and the ^1H NMR spectrum a large doublet at 3.85 ppm ($^1J_{HC}$ = 146 Hz). For compounds issued from the labelled endoperoxides **6b/c**, the same three structural moieties were identified: **8b/c**, **9b/c**, and **10b/c** (**b/c**, in a 1/1 ratio). As previously, the ^{13}C NMR distribution was analyzed: compounds **8b/c** show two intense peaks at 166.0 and 201.6 ppm, assigned to ^{13}C-1 and ^{13}C-4, **9b/c** at 168.0 and 195.0 ppm assigned to ^{13}C-1 and ^{13}C-6, respectively, and then **10b/c**, at 168.2 and 194.5 ppm likewise assigned to ^{13}C-1 and ^{13}C-6. ^1H and $^1H\{^{13}C\}$ spectra of **8b/c** decoupling experiments show also the same simplification in the coupling constant. The mechanisms proposed in Scheme 6 fit quite well with the ^{13}C distribution analysis and NMR results for compounds **5**, **5a**−**c**. After one-electron transfer from Fe(II) to the endoperoxide bridge and homolytic breaking of the O−O bond, O-centered radical **I** rearranges to give carbon-centered radical **II**, a tertiary *gem*-dimethyl carbon radical (more stable). It undergoes 5-*exo-trig* cyclization to give a new radical **III**, α to the five ring structure obtained. When **III** adds to the carbonyl, a small three-membered cycle **IV** is formed that finally leads to a stabilized radical **V** as a result of the electron-withdrawing effect of the COOH function. Acetone elimination and Fe(II) expulsion afford the acid **7**.

The same pathway can be proposed for the Fe(II) reduction of the methylated endoperoxides, **6**, **6a**, and **6b/c**, to explain the formation of the esters **8**, **8a**, and **8b/c** (Scheme 5). As previously, a single-electron transfer from Fe(II) to the O−O bridge occurs first inducing the homolytic breakage of the peroxidic function and then the formation of a *gem*-dimethyl carbon radical. The same sequence of rearrangements finally led to the cyclohexenyl esters **8**, exactly as acid **7** was obtained from G3. However, when compared to G3 reduction, two other compounds were identified, five-ring lactones **9** and **10**, which are systematically obtained in the same relative proportions. The *gem*-dimethyl radical **II** seems to be more reactive with R = OH than with R = OMe, allowing in the latter case an intermolecular disproportionation leading after lactonization to identified compounds **9** and **10**.

Reduction of **11** gave only one compound and combines the elements of the previous pathways: formation of a dioxo-cyclohexenyl part and of a five-ring lactone to give the bicyclic structure **12.** The results are consistent with the following steps (Scheme 6): formation, as previously described, of the oxygen radical **I′**, leading to the fluoro radical **II′**, then a rapid 5-*exo-trig* cyclization, lactonization, and elimination of hydrogen fluoride and Fe(II) to afford bicyclic lactone **12**.

Finally if we compare the results for reduction of the three endoperoxides, the role of the C-centered radicals ($\mathbf{II}_{OH}$, $\mathbf{II}_{OMe}$, or **II′** for the fluoro compound, Schemes 6−8) seems to be determinant. Depending on its stability, the 5 *exo-trig* cyclization will occur immediately or later. Formation of **12** is very rapid, whereas it is slower for **7** and slower again for **8**, since the radical can exchange hydrogen in the disproportionation reaction giving lactones **9** and **10.** This means that the $\mathbf{II}_{OMe}$ radical is less reactive than $\mathbf{II}_{OH}$, the **II′** radical being the most reactive of the three. This observation could explain why **6** presents greater antimalarial activity in vitro, compared to **5** and **11**. Only in the first case, in the biological reduction of endoperoxide **6**, a C-centered radical could live long enough to combine with heme (or to act on proteins) or generate radical oxygen species within the parasitized erythrocyte,[18] a reaction that will be impeded when the intramolecular 5 *exo-trig* cyclization occurs rapidly, a reason for **11** to be inactive and **5** far less active than **6**. The alkylating properties of the C-centered radical are based on a good balance between stability and reactivity. Biological properties could be improved by avoiding this 5 *exo-trig* cyclization. It means trying to hydrogenate the double bond or add a nucleophilic group to it. Work is in progress along these lines and will guide design of new antimalarial endoperoxides, even if we have to be careful in the correlation between in vitro chemical reactivity and in vivo antimalarial activity.[28]

Experimental Section

General Procedure for Endoperoxide Reduction. To endoperoxide (0.12 mmoles) dissolved in 1 mL of degassed acetonitrile is added $FeSO_4$ (31 mg, 0.12 mmoles) first dissolved in 1 mL of degassed water. After 24 h, CH_3CN is evaporated, and the water is lyophilized. Crude mixture is passed through Chelex resin to get rid of the iron.

Reduction of Compound 5. After purification on a Hyperprep C18 column (250 × 20) with water/methanol: 80/20, 15 mL/mn, a major product **7** is obtained in 82% yield from **5**. Data for **7**: *m/z* (electrospray negative mode) 209 (M−H, 100%) 165 (M−H−CO_2); ν_{max} (KBr)/cm^{-1} 3751, 3398, 1674, 1461, 1381, 1105; δ_H (500 MHz; CD_3OD; Me_4Si) 1.33 (6H, s, 10 and 11-Me), 1.53 (6H, s, 8 and 9-Me), 6.53 (1H, s, 3-H); δ_C (135.6 MHz; CD_3OD; Me_4Si) 22.7 (C10−11), 25.1 (C8−9), 47.7 (C7), 58.3 (C5), 122.0 (C3), 158.6 (C2), 169.9 (C1), 201.7 (C4), 215.1 (C6). Data for **7a**: δ_H 1.33 (6H, s, 10 and 11-Me), 1.53 (6H, d, $^3J_{HC}$ = 5 Hz, 8 and 9-Me), 6.53 (s, 1H, 3-H); δ_C 158.6 (C2). Data for **7b/c**: δ_H 1.33 (s and d, $^3J_{HC}$ = 5 Hz, 6H, 10 and 11-Me), 1.53 (s, 6H, 8 and 9-Me), 6.53 (1H, s and d, $^3J_{HC}$ = 10 Hz, 3-H); δ_C 169.9 (C1), 201.7(C4).

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Supporting Information Available: General procedure for endoperoxide reduction; NMR spectra for compounds **7**, **8**, **9**, **10**, **12**, and labelled compounds; HPLC chromatograms determining purity of compounds **7**−**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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