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The Functionalisation of Saturated Hydrocarbons—XXX. Model Studies on the Mechanism of Some Oxygenases

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Abstract—In harmony with our studies on the activation of hydrocarbons by Gif chemistry, we have, in the first part of this paper, studied the mechanism of the lipoxygenase enzymes using soybean lipoxygenase as a model. We have shown with trimethyl phosphite that no free radical is released by the enzyme. In a second part, we have studied the mechanism of the α-ketoglutarate dependent enzymes and shown evidence for a mechanism involving the reduction of an intermediate hydroperoxide by the α-ketoglutaric acid.

Gif chemistry[1] (Scheme I) converts saturated hydrocarbons selectively into ketones under mild conditions (pyridine–acetic acid at room temperature and with a pH near to neutrality). Two intermediates have been detected, (A) and (B). The latter has been thoroughly characterized as the corresponding hydroperoxide.[2] Intermediate (A) is converted into the hydroperoxide (B) by oxygen and into many other derivatives by selective trapping.[3] Many experiments have shown that (A) is not a carbon radical.[4,5] We have postulated it to be an iron–carbon bond.

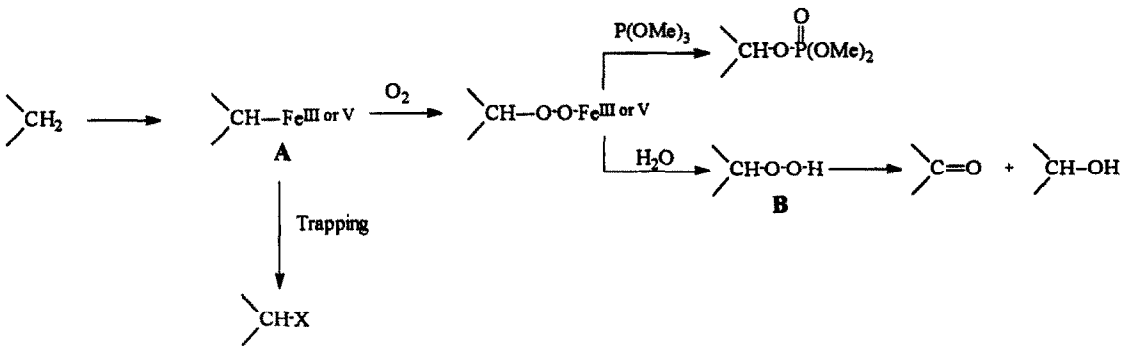
The original inventive idea behind Gif chemistry was that the microorganisms which oxidized metallic iron and Fe^{II} to ferric oxide also oxidized the saturated hydrocarbons present at the time the blue–green algae started to make oxygen and changed the world from anaerobic to aerobic.

There are a number of important non-heme enzymes which oxidize unactivated -CH_2- groups to -CHOH-. Methane monooxygenase[6] and proline hydroxylase[7] are two important examples. The former could, in principle, provide an environmentally acceptable procedure for the oxidation of simple saturated hydrocarbons to useful aliphatic alcohols.[8] The latter, proline hydroxylase, is a vital enzyme for mammalian life since it converts proline residues into hydroxy-proline residues, en route for the synthesis of collagen. If Gif chemistry provides a model for non-heme oxidation enzymes, then these enzymes should also be making the analogs of intermediates (A) and (B). Specifically the hydroxylation reaction should pass through a hydroperoxide (B). We plan to investigate this possibility. We have started with an enzyme that does indeed make a hydroperoxide from oxygen with catalysis provided by iron.

Lipoxygenases[9] are mononuclear non-heme iron enzymes which catalyze the peroxidation of fatty acids containing a skipped 1,4-diene system to give a 1,3-diene-4-hydroperoxide system.

These enzymes play an important role in polyunsaturated fatty acid metabolism in both plants and animals. In the mammalian kingdom, for example, lipoxygenase is involved in the first step in the conversion of arachidonic acid into leukotrienes.[10,11]

The resting state of the lipoxygenase enzymes contains Fe^{II},[12] but the active species is formed with a lag time[13] which is considered to be due to the oxidation of Fe^{II} to Fe^{III}. Two mechanisms have been proposed in the literature. The first[11] (Scheme II) shows that the substrate **1** is actived by internal electron transfer from a group X (probably X=N) to give a species X·+ which abstracts a hydrogen atom from the CH_2 of the skipped diene to form the radical **3**. This then affords the radical **4** by reaction with triplet oxygen. Electron transfer from Fe^{II} coupled with proton transfer from XH+ then gives the reaction product **5**.



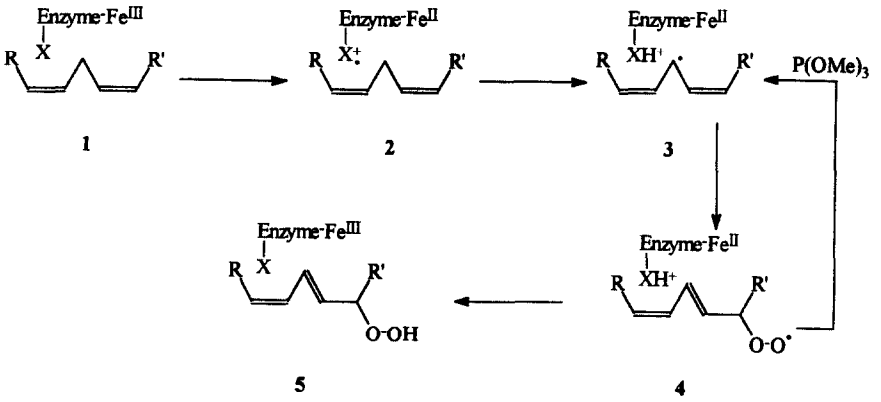
Scheme I.

An alternative mechanism favored by Corey[14] (Scheme III) postulates the formation of an iron–carbon bonded species **6**. The insertion of oxygen into the iron–carbon bond affords an iron peroxo species **7** which by hydrolysis affords **5**.

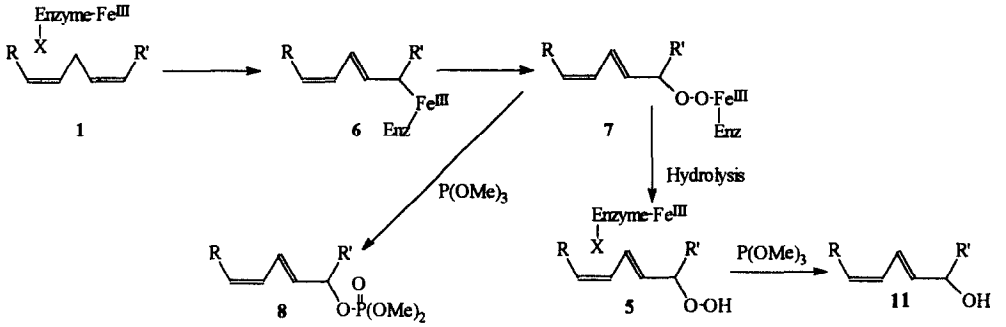
Trimethyl phosphite has played an important role in unraveling the intricacies of Gif chemistry.[2,15] This reagent reduces a hydroperoxide rapidly and quantitatively to an alcohol. It reacts equally rapidly with a hydroperoxyl radical like **4** to reduce it by oxygen transfer to an alkoxy radical which, by the same mechanism, is reduced to the carbon radical **3**. Thus, the carbon radical **3** with oxygen would become a catalyst for the conversion of trimethyl phosphite to trimethyl phosphate. Trimethyl phosphite reacts in a different way with an Fe^{III}-peroxyl species like **7** and affords, in an Arbusov reaction, the corresponding phosphate **8**.[15]

Thus, in principle, the addition of trimethyl phosphite will distinguish between Schemes II and III. To the best of our knowledge trimethyl phosphite has not been used as a reagent in iron based oxidation chemistry by enzymes so the first step of our investigation was to show that the enzyme soybean lipoxygenase[16] (EC 1.13.11.12, purchased from SIGMA) was not simply inactivated by the reagent.

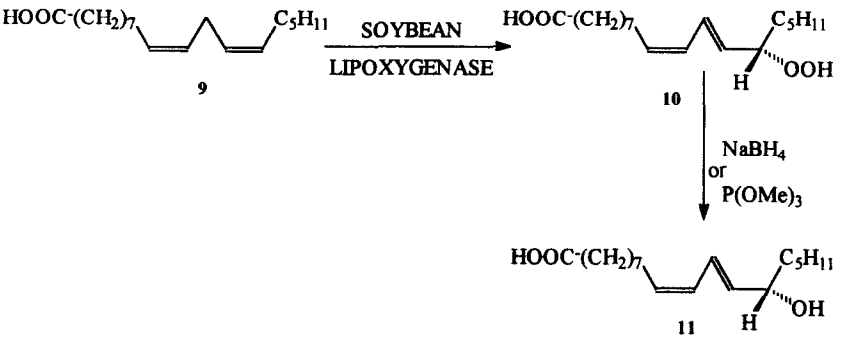
Linoleic acid **9** was converted by the enzyme (Scheme IV) under standard conditions to the known hydroperoxide **10**. Reduction of the latter with sodium borohydride gave the corresponding acid **11** (coriolic acid) which was fully characterized as its methyl ester. Similarly the reduction of **10** with excess trimethyl phosphite gave **11** quantitatively. We were now ready for the critical experiment. The addition of an excess of trimethyl phosphite (up to 100 times more) to linoleic acid before the addition of the enzyme gave exclusively coriolic acid at the same rate and with the same yield as that observed for the formation of the hydroperoxide **10**.



Scheme II. X = source of electron transfer and radical cation formation, R,R' = chain extensions.



Scheme III.



Scheme IV.

So not only did the trimethyl phosphite have no inhibitory effect on the enzyme, it also did not provide evidence in support of either Scheme II (intermediate **4**) or Scheme III (intermediate **7**). Certainly the peroxyl radical **4** is not released from the enzyme and the species **7** is so bound that it is inaccessible to the trimethyl phosphite. There have been a number of reports of free carbon or free peroxyradicals in the lipoxygenase oxidations in the literature.[17] In many cases the radicals have been detected in anaerobic conditions and in absence of careful quantification. These results seem to be misleading.

A number of non-heme iron based oxidation enzymes require α-ketoglutaric acid as a co-substrate.[18] It is generally accepted that this acid is involved in the activation of the iron so that saturated C–H bonds can be hydroxylated. An alternative possibility is that the α-ketoglutaric acid simply reduces an initially formed hydroperoxide to the alcohol actually isolated.

If α-ketoglutaric acid is added to the lipoxygenase catalyzed oxidation of linoleic acid either before the enzyme is added or after the enzymatic reaction, coriolic acid is again formed quantitatively. NMR Studies in pyridine-d_5 showed that H_2O_2 and *t*-butylhydroperoxide both reacted rapidly at room temperature with α-ketoglutaric acid to give succinic acid and carbon dioxide. Similarly cyclohexyl hydroperoxide, prepared as described by Williams,[19] gave cyclohexanol, succinic acid and carbon dioxide. These experiments show that the hydroperoxide linkage is indeed reduced efficiently by α-ketoglutaric acid (Scheme V).

The family of α-ketoglutarate dependent enzymes mentioned above includes several enzymes like ε-*N*-trimethyl lysine hydroxylase and γ-butyrobetaine hydroxylase,[20] which are involved in the biosynthesis of L-carnitine; prolyl 3-hydroxylase, prolyl 4-hydroxylase[7] and lysyl hydroxylase,[21] which are involved in the biosynthesis of collagen. Thymine 7-hydroxylase,[22] pyrimidine deoxyribonucleoside 2'-hydroxylase, deoxyuridine 1'-hydroxylase,[23] deoxyuridine 2'-hydroxylase[23] and the enzyme deacetoxy/deacetylcephalosporin C synthase (DAOC/DACS) which is responsible for the ring expansion of penicillin N, are also α-ketoglutarate dependent.[24]

α-Ketoisocaproate dioxygenase[25] and *p*-hydroxyphenylpyruvate hydroxylases[26] are two special cases in which the α-ketoacid function is in the substrate.

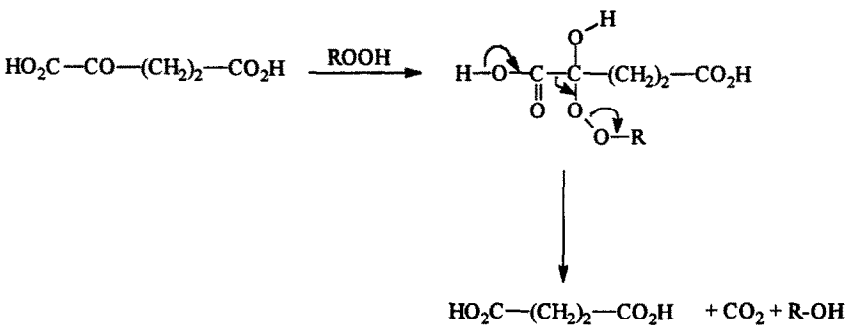
All these enzymes are characterized by the fact that they need ferrous iron, molecular oxygen and α-ketoglutaric acid as a cofactor during the oxidation process. Ascorbic acid is also required. The α-ketoglutaric acid is stoichiometrically decarboxylated to succinate and CO_2, in the reaction with one atom of the O_2 molecule being incorporated into the succinate and the other into the OH group of the substrate. These enzymes are able to perform an enzyme dependent decarboxylation of the ketoglutaric acid in the absence of the substrate,[27] the presence of ascorbic acid is required for this process.[28] Much work has been done on these enzymes and different mechanisms have been proposed in the literature, but at this time none has been clearly demonstrated.

Several interpretations of how α-ketoglutarate and oxygen can activate Fe^{II} have been made. We note specially the ideas of Siegel[29] which can be summarized as in Scheme VI. It is difficult to see in this Scheme why the loss of CO_2 has anything to do with activation of the Fe^{IV}.

A modification of this Scheme would be to have the ascorbic acid reduce the oxygen to superoxide by hydrogen atom transfer. Reaction with Fe^{II} would then lead to the species Fe^{III}-O–OH which then can afford Fe^{V}=O as in Gif chemistry. In this case iron–carbon bond formation would follow. Reduction of Fe^{V} to Fe^{III} and insertion of oxygen would then afford the hydroperoxide which would then be reduced to the alcohol by the α-ketoglutaric acid.

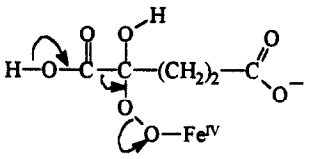
Based on P_{450} enzyme chemistry, the reaction of Fe^{II} compounds with oxygen could also be considered. However, with Fe^{II} non-porphyrin models this is a slow reaction compared with the fast reaction with superoxide.

Another mechanism involving a peroxysuccinic acid formed from the α-ketoglutaric acid and oxygen that can react after with the substrate has been proposed by Hamilton[30] (Scheme VII). Since this per-acid cannot be used in place of α-ketoglutaric acid this proposal seems improbable. An aliphatic hydrocarbon is also normally stable toward mild peracids.

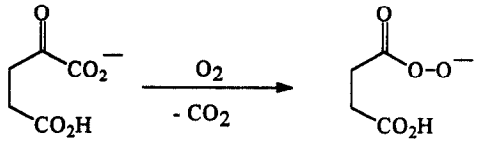


R = H, ^{t}Bu or C_6H_{11}

Scheme V.



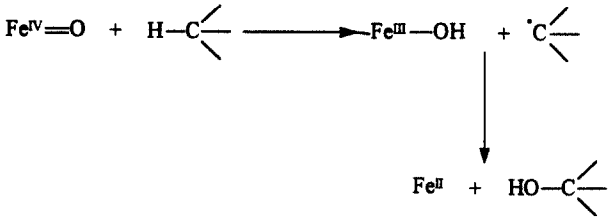
Scheme VI.



Scheme VII.

In the case of the DAOC/DACS enzyme, Baldwin[24f] proposed a mechanism based on different experiments using ^{18}O and $H_2{}^{18}O$. This mechanism involves the insertion of an iron oxo species [Fe^{IV}=O] into the C–H bond followed by a ligand coupling between the alkyl and the hydroxyl group. The formation of the iron oxo species is described as in Scheme VI by action of oxygen and the α-ketoglutaric acid.

An alternative mechanism has been proposed in the case of the γ-butyrobetaine hydroxylase[31] This involves a homolytic carbon–hydrogen bond cleavage to yield a carbon radical.



Scheme VIII.

The possible formation of a hydroperoxide has already been suggested.[32] As pointed out by Hamilton[30] the problem with this mechanism was to explain how the hydroperoxide can be formed from an unreactive aliphatic substrate. In Gif chemistry the intermediate during the oxidation process is the hydroperoxide derived from the alkane, this intermediate giving rise to alcohol and ketone.

To show that this mechanism might be applicable we first (as described above) showed that α-ketoglutaric acid is able to reduce hydroperoxides. Then we verified that ascorbic acid is able to activate the Fe^{II} as described by Udenfriend.[33] We oxidized cyclododecane in a mixture of pyridine–acetic acid (the Gif chemistry solvent) with a catalytic amount of Fe^{II} in presence of some ascorbic acid and obtained 11 % of oxidation based on the starting material (Table 1, entry 1). In a second experiment, we showed that in the same solvent mixture, α-ketoglutaric acid alone is not able to 'activate' the iron to oxidize the cyclododecane (Table 1 entry 2).

Since Gif chemistry involves the formation of hydroperoxides we examined the effect of α-ketoglutaric acid on an Fe^{II}(cat.)–$Zn°$–O_2 reaction. The latter involves the reduction of oxygen to superoxide which then reacts with Fe^{II}. However, this system at once reduced the α-ketoglutaric acid to the corresponding alcohol which was characterized as its dimethyl ester.

The alternative experiment using Fe^{III}(cat.)–H_2O_2–picolinic acid also failed when we added α-ketoglutaric acid at the beginning of the reaction because the hydrogen peroxide reacted immediately with α-ketoglutaric acid to give succinic acid. On the other hand, we were able to change the ketone:alcohol ratio by quenching the reaction with oxalic acid (10 minutes after the addition of the hydrogen peroxide) and then adding the α-ketoglutaric acid to reduce the hydroperoxide formed during the oxidation. The ratio ketone:alcohol in this case was the same as when using triphenyl phosphine to reduce the hydroperoxide, showing again the ability of α-ketoglutaric acid to reduce alkyl hydroperoxides, (Table 2).

Recent elegant work by Baldwin[24f] and his collaborators using $^{18}O_2$ oxygen and H_2O^{18} has shown that exchange of water with the intermediate iron oxenoid species complicates the discussion of β-lactam based enzyme

Table 1. Oxidation of the cyclododecane by Fe^{II} (2 mmol) with ascorbic acid or α-ketoglutaric acid.

ENTRY	ASCORBIC ACID (mmoles)	KETOGLUTARIC ACID (mmoles)	CYCLODODECANE (mmoles)	CYCLODODECANONE (mmoles)	CYCLODODECANOL (mmoles)
1	10	---	4.5	0.4	0 17
2	---	20	4.95	0	0

Table 2.

ENTRY	WORK UP	CYCLODODECANONE (mmoles)	CYCLODODECANOL (mmoles)
1	oxalic acid	0.056	trace
2	oxalic acid + $P\Phi_3$	trace	0.056
3	ketoglutaric acid + oxalic acid	trace	0.057

mechanism. There is no clear evidence that a hydroperoxide is an intermediate in any of the enzymatic reactions studied. However, the fact that in our experiments with α-ketoglutaric acid no activation could be effected without the addition of a reducing agent suggests that superoxide, not oxygen, is involved. If this is true then reaction with Fe^{II} will lead to Fe^{III}–O–OH and thence to Fe^{V} as in Gif chemistry.[1,2] Hitherto the β-lactam based enzymes have been considered to involve Fe^{IV} oxenoid species.[24]

In conclusion we have shown that the mechanism of the enzyme lipoxygenase does not involve the release of a free carbon radical.

The oxygenation has a cage-like mechanism and trimethyl phosphite does not have access to the cage.

We have been unable to secure any evidence that Fe^{II}, α-ketoglutaric acid and oxygen give an activated species that can attack saturated hydrocarbons. However, hydrogen atom transfer from ascorbic acid without α-ketoglutaric acid does permit activation and formation of hydroperoxide. The reduction of the latter by the α-ketoglutaric acid is very fast. We conclude that non-heme enzymes may follow a Gif-type mechanism in certain cases.

Experimental Section

Gas chromatography–mass spectrometric analyses were performed on a Hewlett–Packard 5890 series II gas chromatograph equipped with a Hewlett–Packard 5971 mass-selective detector. Gas chromatography studies were performed on a Hewlett–Packard 5890 series II instrument equipped with a hydrogen flame ionization detector using nitrogen as a carrier gas. The column used was a DB-WAX capillary column from J&W Scientific (30m; 0.32 mm i.d.; 25 μm film thickness). ^1H NMR and ^{13}C NMR experiments were carried out at room temperature on a Varian XL 200 NMR spectrometer operating at 200 MHz and 50 MHz respectively using 5 mm tubes and tetramethylsilane as an internal standard. UV spectra were recorded on a Beckman DU-7 spectrophotometer using a 1 cm light path cell. Optical rotation measurements were performed using a JASCO model DIP 360 digital polarimeter.

Oxidation with lipoxygenase

For all reactions, the lipoxygenase (EC 1.13.11.12) purchased from SIGMA (Lipoxygenase Type IV from soybean, suspension in 2.3 M $(NH4)_2SO_4$) was used. All the reactions were carried out using the 'low ethanol method' in a 0.2 M borate buffer as described by SIGMA.[34] The specific activity of the enzymes was determined to be 520,000 units per mg of protein (one unit causes an increase in A_{234} of 0.001 per minute at pH 9.0 at 25 °C, when linoleic acid was used as substrate. Reaction Volume = 3.0 mL (1 cm light path)).

Determination of the activity of the enzyme

The specific activity of the commercial enzyme was determined by UV as described by SIGMA[34] looking at the variation of the absorbance at 234 nm during the oxidation of the linoleic acid by the lipoxygenase at pH 9.0 in a borate buffer.

$$\text{Units / mg solid} = \frac{\Delta A_{234}\text{ / minute}}{\text{0.001 mg Enzyme / 3 ml Reaction Volume}}$$

General procedure for oxidation with the enzyme

50 μL of linoleic acid (0.16 mmol, 45 mg) and 50 μL of ethanol were added to 50 mL of a 0.2 M borate buffer pH 9.0 under vigorous stirring. To the solution thus prepared 100 μL of the commercial enzyme were added and the reaction was gently stirred in a 125 mL Erlenmeyer under air at room temperature. The reaction was followed by TLC (an aliquot of the reaction was acidified, extracted with ether and then esterified by CH_2N_2). After 2 h the TLC showed the disappearance of the starting material and the formation of a new product. The reaction mixture was then diluted with 100 mL of ether and carefully acidified to pH 4 at 0 °C with H_2SO_4 25 %. The organic phase was decanted, dried over $MgSO_4$ and evaporated under reduced pressure.

Synthesis of a pure sample of the 13(S)-hydroxy-9(Z), 11(E)-octadecadienoic acid (coriolic acid)

The reaction was carried out as described in the general experiment above leading to the 13(*S*)-hydroperoxy-9(*Z*), 11(*E*)-octadecadienoic acid as is well known.[9b] ^1H NMR ($CDCl_3$): 6.6 (dd, 1H, H_{11}), 6 (t, 1H, H_{10}); 5.6 (dd, 1H, H_{12}); 5.5 (dt, 1H, H_9); 4.4 (q, 1H, H_{13}), 2.27 (t, 2H, H_2), 2.14 (q, 2H, H_8); 1.8–0.8 (m, 21H). ^{13}C NMR ($CDCl_3$): 13.99, 22.48, 24.93, 27.67, 28.86, 28.89, 29.00, 29.05, 29.33, 31.71, 32.49, 33.96, 86.80, 127.55, 130.00, 131.21, 133.85, 179.82.

The crude hydroperoxide (30 mg) was dissolved in 50 mL of methanol and 1.5 equivalent of sodium borohydride were added at 0 °C. The reaction was stirred at room temperature for 30 min and then the solvent was evaporated *in vacuo*. The solid product was acidified to pH 4 with H_2SO_4 25 % and the resulting solution extracted with ether leading to the almost pure coriolic acid. The product was then esterified by action of a solution of CH_2N_2 in ether and purified by column chromatography (SiO_2, Et_2O). The coriolic acid was obtained optically pure, as shown by the optical rotation ($[\alpha]^{24}D$ = +6.0 (c = 1.6, hexane) Lit.[36] $[\alpha]^{24}D$ = +6.0 (c = 1.6, hexane)) with a yield of 90 % from the linoleic acid. The ^1H NMR and the ^{13}C NMIR were in agreement with the literature for the coriolic acid methyl ester.[35] ^1H NMR ($CDCl_3$): 6.46 (dd, 1H, H_{11}), 5.95 (t, 1H, H_{10}), 5.64 (dd, 1H, H_{12}), 5.41 (dt, 1H, H_9), 4.15 (q, 1H, H_{13}), 3.65 (s, 3H, COOMe), 2.27 (t, 2H, H_2), 2.14 (q, 2H, H_8), 1.8–0.8 (m, 21H). ^{13}C NMR ($CDCl_3$): 13.98, 22.46, 22.54, 24.82, 25.06, 27.59, 28.87, 28.98, 31.34, 31.72, 32.55, 37.24, 51.39, 72.80, 125.63, 127.77, 132.67, 135.91, 174.28.

Synthesis of coriolic acid by reduction with trimethyl phosphite

The reaction was carried out as described in the general

procedure except for the addition of 30 equivalents of trimethyl phosphite before (or two hours after) the addition of the enzyme. In both cases coriolic acid was obtained quantitatively. The addition of 100 equivalents of trimethyl phosphite instead of 30 equivalents gave the same result. The product was also esterified by treatment with a solution of CH_2N_2 in ether and purified by column chromatography (SiO_2, Et_2O) to remove salts and the excess trimethyl phosphite. The ^1H NMR, ^{13}C NMR and the optical activity ($[\alpha]^{24}D = +6.02$ (c = 1.6, hexane)) were recorded showing in each case that coriolic acid methyl ester had not racemized.

Synthesis of coriolic acid by reduction with α-ketoglutaric acid

The reaction was carried out as described in the general procedure except for the addition of 1.5 equivalents of α-ketoglutaric acid before (or two hours after) the addition of the enzyme. In both cases coriolic acid was obtained quantitatively. The product was also esterified by treatment with a solution of CH_2N_2 and purified by column chromatography (SiO_2, Et_2O). The ^1H NMR, ^{13}C NMR and the optical activity ($[\alpha]^{24}D = +6.0$ (c = 1.6, hexane)) were recorded showing in each case that coriolic acid had not racemized.

Reaction with Fe^{II} and ascorbic acid (Table 1, entry 1)

A solution of cyclododecane (5 mmol, 0.842 g) in a mixture of pyridine (30 mL) acetic acid (3 mL) containing $FeCl_2 \cdot 4H_2O$ (2 mmol, 0.4 g) was placed in a 125 mL Erlenmeyer flask open to air and stirred well. Ascorbic acid (10 mmol, 1.76 g) was added to the solution prepared above, and the reaction was stirred overnight.

Work-up procedure. 1 mL of the reaction mixture was placed in a test tube. Ether (10 mL) and a solution of an internal standard (naphthalene) were added. Then the solution was chilled and acidified to pH 2 by dropwise addition of 25 % (v/v) H_2SO_4. The mixture was then shaken vigorously. The ether layer was separated and washed with 3 mL of a solution of $NaHCO_3$ sat. The organic phase was decanted, dried ($MgSO_4$) and analyzed by gas chromatography.

Reaction with Fe^{II} and α-ketoglutaric acid (Table 1, entry 2)

The reaction was carried out as described for ascorbic acid except that α-ketoglutaric acid (20 mmol, 2.92 g) was used instead of the 10 mmol of ascorbic acid. The reaction was sumitted to the same work-up procedure described above and analyzed by GC. This procedure was repeated with quantitites of $FeCl_2 \cdot 4H_2O$ varying from 0.25 to 3 mmol. For each experiment no oxidation of the cyclododecane was detected by GC.

α-Ketoglutaric acid and Fe^{II}(cat.)–Zn^0–O_2

To a solution containing cyclododecane (5 mmol, 0.842 g), $FeCl_2 \cdot 4H_2O$ (0.25 mmol, 0.05 g) and α-ketoglutaric acid (10 mmol, 1.46 g) in a mixture of pyridine (30 mL) and acetic acid (3 mL) was added zinc powder (10 mmol, 1.3 g). The reaction was stirred under air at room temperature overnight. Without the α-ketoglutaric acid it takes all the night for the zinc powder to disappear but with the α-ketoglutaric acid there was no more zinc after 30 min. The normal work-up was performed on a 1 mL aliquot of the reaction mixture (see Fe^{II} and ascorbic acid). No oxidation of the cyclododecane was found. The remaining reaction mixture was evaporated *in vacuo*, the product dissolved in ether and washed at 0 °C with H_2SO_4 25 % and with brine. The organic phase was then dried ($MgSO_4$) and evaporated *in vacuo*. The crude product was then esterified by treatment with a solution of CH_2N_2 in ether and purified by column chromatography (SiO_2, Et_2O). The product was characterized by NMR and GC/MS as dimethyl 2-hydroxy-glutarate[37] (yield 95 %). ^1H NMR ($CDCl_3$): 1.93 (dtd, 1H, H3), 2.17 (dtd, 1H, H3), 2.43 (ddd, 1H), 2.5 (dt, 1H, H4), 2.85 (m, 1H, OH), 3.67 (s, 3H, CH3), 3.79 (s, 3H, CH3), 4.23 (dd, 1H, H2) ^{13}C NMR ($CDCl_3$): 28.86, 29.03, 51.29, 52.06, 69.13, 173.3, 174.56.

α-Ketoglutaric acid and Fe^{III}(cat.)–H_2O_2-picolinic acid (Table 2)

Cyclododecane (5 mmol, 0.842 g), $FeCl_3 \cdot 6H_2O$ (0.2 mmol, 0.05 g) and picolinic acid (0.6 mmol, 0.074 g) were dissolved in pyridine (30 mL) and acetic acid (3 mL). H_2O_2 (10 mmol, 1 ml 30 % solution in water) was then added to start the reaction, and after 10 min 3 aliquots (1 mL) of the reaction were taken.

In the first one, oxalic acid, which is known to complex the iron and stop the reaction, (5 mmol, 0.45 g) was added and after 5 min the normal work-up (see Fe^{II}-ascorbic acid experiment above) was performed. Cyclododecanone (0.056 mmol) was formed (entry 1).

In the second one, oxalic acid (5 mmol, 0.45 g) and triphenyl phosphine (5 mmol, 1.31 g), which is known to reduce hydroperoxides, were added and the normal work-up performed after 30 min. Cyclododecanol (0.055 mmol) was formed (entry 2).

In the third one, oxalic acid (5 mmol, 0.45 g) and α-ketoglutaric acid (5 mmol, 0.73 g) were added and the work-up was performed after 30 min. Cyclododecanol (0.057 mmol) was determined (entry 3).

α-Ketoglutaric acid and H_2O_2

In an NMR tube containing 1 g of pyridine-d_5 and α-ketoglutaric acid (1 mmol) was added quickly 1 mmol of H_2O_2 (30 % in water). The ^1H NMR spectrum was recorded every 5 min showing the disappearance of the α-ketoglutaric acid and the appearance of succinic acid. By comparison of the integration of the peak at 2.8 ppm (corresponding at 2 protons of the α-ketoglutaric acid) and the peak at 2.2 ppm (corresponding to 4 protons of the succinic and 2 protons of the α-ketoglutaric acid) the half

life of the α-ketoglutaric acid was determined to be less than 15 min. The same experiment made in D_2O had the same half life.

α-Ketoglutaric acid and tert-butyl hydroperoxide

In an NMR tube containing 1 g of pyridine-d_5 and 1 mmol of α-ketoglutaric acid (0.146 g) was added quickly 1 mmol of *tert*-butyl hydroperoxide (90 %). The ^1H NMR spectrum was recorded every 5 min showing the disappearance of the a-ketoglutaric acid and the appearance of succinic acid. By comparison of the integration of the peak at 2.8 ppm (corresponding to 2 protons of the α-ketoglutaric acid) and the peak at 2.2 ppm (corresponding at 4 protons of the succinic and 2 protons of the α-ketoglutaric acid) the half life of the α-ketoglutaric acid was determined to be less than 15 min. The same experiment made in D_2O had the same half life.

α-Ketoglutaric acid and cyclohexyl hydroperoxide

A pure sample of cyclohexyl hydroperoxide was preparted as described elsewhere.[2,18]

To a solution containing cyclohexyl hydroperoxide (1 mmol, 0.116 g) in pyridine-d_5 (1 g) was quickly added α-ketoglutaric acid (1 mmol, 0.146 g). The reaction was monitored by ^{13}C NMR looking at the disappearance of the peak corresponding to the carbon of the hydroperoxide (82.2 ppm) and the appearance of the corresponding alcohol (69.07 ppm). the spectrum was recorded every 5 min showing that the half life of the cyclohexyl hydroperoxide was less than 15 min.

α-Ketoglutaric acid and Fe^{II} (cat.)–KO_2

Cyclohexane (50 mmol, 4.2 g), $FeCl_2 \cdot 4H_2O$ (2.5 mmol, 0.49 g) were dissolved in pyridine (30 mL) and acetic acid (5 mL). KO_2 (10 mmol, 0.71 g) was then added in 4 portions over 4 h and the reaction was stirred over night under argon.

After work-up and analysis by GC of the reaction, 0.63 mmol of cyclohexanone were produced. The same reaction performed in the presence of 20 mmol α-ketoglutaric acid did not give any oxidation products due to the fast reaction between the oxidant and α-ketoglutaric.

α-Ketoglutaric acid and KO_2

In a solution containing pyridine-d_5 (3 mL), TFA (0.3 mL) and 1 mmol of α-ketoglutaric acid (0.146 g) was added 1 mmol of KO_2 (0.071g). The reaction was stirred over night under argon. The solution was then filtered and the ^1H NMR spectrum was recorded showing the conversion of 45 % of α-ketoglutaric acid into succinic acid, by comparison of the integration of the peak at 2.8 ppm (corresponding at 2 protons of the α-ketoglutaric acid) and the peak at 2.2 ppm (corresponding at 4 protons of the succinic and 2 protons of the α-ketoglutaric acid).

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References

1. Barton, D. H. R.; Doller, D. *Acc. Chem. Res.* **1992**, *25*, 504–512.

2. Barton, D. H. R.; Bévière, S. D.; Chavasiri, W.; Csuhai, E.; Doller, D.; Liu, W.-G. *J. Am. Chem. Soc.* **1992**, *114*, 2147–2156.

3. Barton, D. H. R.; Bévière, S. D.; Chavasiri, W.; Doller, D.; Hu, B. *Tetrahedron Lett.* **1993**, *34*, 1871–1874.

4. Barton, D. H. R.; Csuhai, E.; Doller, D. *Tetrahedron*, **1992**, *48*, 9195–9206.

5. Barton, D. H. R.; Chavasiri, W; Hill, D. R.; Hu, B. *New J. Chem.* in press.

6. (a) Colby, J.; Dalton, H. *Biochem. J.* **1978**, *171*, 461–468; (b) Fox, B. G.; Surerus, K. K.; Münck, E.; Lipscomb, J. D. *J. Biol. Chem.* **1988**, *263*, 10553–10556; (c) Fox, B. G.; Borneman, J. G.; Wackett, L. P.; Lipscomb, J. D. *Biochemistry*, **1990**, *29*, 6419–6427; (d) see also Reference 8.

7. (a) Cardinale, G. J.; Udenfriend, S. *Adv. Enz.* **1974**, *41*, 245–301; (b) Kivirikko, K. I.; Myllylä, R.; Pihlajaniemi, T. *FASEB Journal* **1989**, *3*, 1609–1617; (c) Tuderman, L.; Myllyla, R.; Kivirikko, K. I. *Eur. J. Biochem.* **1977**, *80*, 341–348; (d) Vuori, K.; Pihlajaniemi, T.; Marttila, M.; Kivirikko, K. I. *Proc. Natl Acad. Sci. U.S.A.*, **1992**, *89*, 7467–7470; (e) Kivirikko, K. I.; Myllyla, R.; Pihlajaniemi, T. *FASEB J.* **1989**, 1609–1617.

8. Green, J.; Dalton, H. *J. Biol. Chem.* **1989**, *264*, 17698–17703.

9. (a) Veldink, G. A.; Vliegenthart, J. F. G. *Adv. Inorg Biochem.* **1984**, *6*, 139–161; (b) Feiters, M. C.; Boelens, H.; Veldink, G. A.; Vliegenthart, J. F. G.; Navaratnam, S.; Allen, J. C.; Nolting, H.-F.; Hermes, C. *Recl. Trav. Chim. Pays-Bas* **1990**, *109*, 133–146.

10. (a) Samuelsson, B.; Dahlen, S.-E.; Lindgren, J. A.; Rouzer, C. A.; Serhan, C. N. *Science (Washington D.C.)* **1987**, *237*, 1171–1176.

11. Bioactive molecules, In *Leukotrienes and Lipoxygenases: Chemical, Biological and Clinical Aspects*, Vol. 11, Rokach, J., Ed.; Elsevier, New York, 1989.

12. Kulkarni, A. P.; Mitra, A.; Chaudhuri, J.; Byczkowski, J. Z.; Richards, I. *Biochem. Biophys. Res. Commun.* **1990**, *166*, 417–423.

13. Slappendel, S.; Malmstrom, B. G.; Petersson, L.; Ehrenberg, A.; Veldink, G. A.; Vliegenthart, J. F. G. *Biochem. Biophys. Res. Commun.* **1982**, *108*, 673–677.

14. Corey, E. J.; Nagata, R. *J. Am. Chem. Soc.* **1987**, *109*, 8107–8108.

15. Barton, D. H. R.; Bévière, S. D.; Doller, D. *Tetrahedron Lett.* **1991**, *32*, 4671–4674

16. Christopher, J. P.; Pistorius, E. K; Axelrod, B. *Biochim. Biophys. Acta* **1972**, *284*, 54–62.

17. (a) De Groot, J. J. M. C.; Garssen, G. J.; Vliegenthart, J. F. G.; Boldingh, J. *Biochim. Biophys. Acta* **1973**, *326*, 279–284; (b) Chamulitrat, W.; Mason, R. P. *J. Biol Chem.* **1989**, 264, 20968–20973; (c) Connor, H. D.; Fischer, V.; Mason, R. P. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 614–621; (d) Sekiya, J.; Aoshima, H.; Kajiwara, T.; Togo, T.; Hatanaka, A. *Agric. Biol. Chem.* **1977**, *41*, 827– 832; (e) Nelson, M. J.; Cowling, R. A. *J. Am. Chem. Soc.* **1990**, *112*, 2820–2821; (f) De Groot, J. J. M. C.; Veldink, G. A.; Vliegenthart, J. F. G.; Boldingh, J.; Wever, R.; Van Gelder, B. F. *Biochim. Biophys. Acta* **1975**, *377*, 71–79; (g) Gardner, H. W. In *Autooxidation in Food and Biological Systems*, pp 447–504, Simic M. G.; Karel, M. Eds.; Plenum Press; New York, 1980.

18. Abbott, M. T., Udenfriend, S. *α-Ketoglutarate-Coupled Dioxygenases* In *Molecular Mechanisms of Oxygen Activation*, pp 167–214, Hayaishi, O. Ed.; Academic Press; New York, 1974.

19. (a) Williams, H. R.; Mosher, H. S. *J. Am. Chem. Soc.* **1954**, *76*, 2984–2987; (b) Williams, H. R.; Mosher, H. S. *J. Am. Chem. Soc.* **1954**, *76*, 2987–2990.

20. (a) Lindstedt, G.; Lindstedt, S.; Olander, B.; Tofft, M. *Biochim. Biophys. Acta* **1968**, *158*, 503–505; (b) Ng, S.-F.; Hanauske-Abel, H. M.; Englard, S. *J. Biol. Chem.* **1991**, *266*, 1526–1533; (c) Ziering, D. L.; Pascal, Jr, R. A. *J. Am. Chem. Soc.* **1990**, *112*, 834–838.

21. (a) Jiang, P.; Ananthanarayanan, V. S. *J. Biol. Chem.* **1991**, *266*, 22960–22967; (b) Myllyla, R.; Pihlajaniemi, T.; Pajunen, L.; Turpeenniemi-Hujanen, T.; Kivirikko, K. I. *J. Biol. Chem.* **1991**, *266*, 2805–2810; (c) Murad, S.; Pinnell, S. R. *J. Biol. Chem.* **1987**, *262*, 11973–11978.

22. (a) Abbott, M. T.; Schandl, E. K.; Lee, R. F.; Parker, T. S.; Midgett, R. J. *Biochim. Biophys Acta* **1967**, *132*, 525–528; (b) Holme, E.; Lindstedt, G.; Lindstedt, S.; Tofft, M. *Biochim. Biophys. Acta* **1970**, *212*, 50–57; (c) Thornburg, L. D.; Stubbe, J. *J. Am. Chem. Soc.* **1989**, *111*, 7632–7633.

23. Stubbe, J. *J. Biol. Chem.* **1985**, *260*, 9972–9975.

24. (a) Baldwin, J. E.; Adlington, R. M.; Aplin, R. T.; Crouch, N. P.; Wilkinson, R. *Tetrahedron* **1992**, *48*, 6853–6862; (b) Baldwin, J. E.; Adlington, R. M.; Aplin, R. T.; Crouch, N. P.; Knight, G.; Schofield, C. J. *J. Chem. Soc. Chem. Commum.* **1987**, 1651–1654; (c) Baldwin, J. E.; Adlington, R. M.; Aplin, R. T.; Crouch, N. P.; Schofield, C. J.; Ting H.-H. *J. Chem. Soc. Chem. Commun.* **1987**, 1654–1656; (d) Baldwin, J. E.; Bradley, M. *Chem. Rev.* **1990**, *90*, 1079–1088; (e) Baldwin, J. E.; Adlington, R. M.; Crouch, N. P.; Keeping, J. W.; Leppard, S. W.; Pitlik, J.; Schofield, C. J.; Sobey, W. J.; Wood, M. E. *J. Chem. Soc. Chem. Commum.* **1991**, 768–770; (f) Baldwin, J. E.; Adlington, R. M.; Crouch, N. P.; Pereira, I. A. C. *Tetrahedron* **1993**, *49*, 7499–7518; (g) Cooper, R. D. G. *BioMed. Chem.* **1993**, *1*, 1–17.

25. Han, H.; Pascal, Jr, R.A. *J. Org. Chem.* **1990**, *55*, 5173–5176.

26. Lindblad, B.; Lindstedt, G.; Lindstedt, S. *J. Am. Chem. Soc.* **1970**, *92*, 7446–7449.

27. Counts, D. F.; Cardinale, G. J.; Udenfriend, S. *Proc. Natl Acad. Sci. U.S.A.* **1978**, *75*, 2145–2149.

28. (a) Yu, R.; Kurata, T.; Arakawa, N. *Agric. Biol. Chem.* **1988**, *52*, 721–728; (b) Yu, R.; Kurata, T.; Arakawa, N. *Agric. Biol. Chem.* **1988**, *52*, 729–733.

29. Siegel, B. *Bioorganic Chemistry* **1979**, *8*, 219–226.

30. Hamilton, G. In *Progress in Bioorganic Chemistry*, Vol. 1, pp 83–157, Kaiser, E.; Kezdy, F. Eds.; Wiley; New York, 1971.

31. Englard, S.; Blanchard, J. S.; Midelfort, C. F. *Biochemistry*, **1985**, *24*, 1110–1116.

32. (a) Lindblad, B.; Lindstedt, G., Tofft, M.; Lindstedt, S. *J. Am. Chem. Soc.* **1969**, *91*, 4604–4606; (b) Holme, E., Lindstedt, G.; Lindstedt, S.; Tofft, M. *FEBS Lett.* **1968**, *2*, 29–32; (c) see also References 7 and 30.

33. (a) Udenfriend, S.; Clark, C. T., Axelrod, J.; Brodie, B. B. *J. Biol. Chem.* **1954**, *208*, 731–739; (b) Brodie, B. B.; Axelrod, J.; Shore, P. A.; Udenfriend, S. *J. Biol. Chem.* **1954**, *208*, 741–750; (c) Hamilton, G. A.; Workman, R. J.; Woo, L. *J. Am. Chem. Soc.* **1964**, *86*, 3390–3391

34. Accompanying literature when the enzyme is purchased from SIGMA.

35. (a) Tallent, W. H.; Harris, J.; Wolff, I. A.; Lundin, R. E. *Tetrahedron Lett.* **1966**, *36*, 4329–4334. (b) Tranchepain, I.; Le Berre, F.; Duréault, A.; Depezay, J. C. *Tetrahedron* **1989**, **45**, 2057–2065.

36. Suemune, H.; Hayashi, N.; Funakoshi, K.; Akita, H.; Oishi, T.; Sakai, K. *Chem. Pharm. Bull.* **1985**, *33*, 2168–2170.

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