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Regioselective Hydroxylation in the Production of 25-Hydroxyvitamin D by *Coprinopsis cinerea* Peroxygenase

Esteban D. Babot,[a] José C. del Río,[a] Lisbeth Kalum,[b] Angel T. Martínez,[c] and Ana Gutiérrez*[a]

Monohydroxylated metabolites of vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol), generically known as 25-hydroxycalciferol, are better for several diseases, and other applications, than vitamin D (calciferol). This work describes a novel biotechnological approach for the preparation of 25-hydroxycalciferols, starting from readily available cholecalciferol and ergocalciferol. This approach enables the regioselective (100%) hydroxylation of these compounds (at the C-25 position) under mild and environmentally friendly conditions by using a peroxidase from the fungus *Coprinopsis cinerea* (gene model CC1G_08427T0 from the sequenced genome), which catalyzes monooxygenation with H_2O_2 as the only co-substrate (peroxygenase). Hydroxylation of cholecalciferol and ergocalciferol is a true peroxygenation, as demonstrated by incorporation of ^{18}O from $H_2{}^{18}O_2$ into the products. The peroxygenase has additional advantages related to its recombinant nature, enabling enzyme engineering and low-cost overexpression in an industrial host. Therefore, the peroxygenase is a promising biocatalyst for the production of vitamin D active metabolites.

Introduction

Vitamin D (calciferol) includes a group of liposoluble secosteroids that are essential in the homeostasis of calcium and phosphate through the regulation of hundreds of genes in animal metabolism.[1] Vitamin D deficiency is a great concern for human health because it causes rickets in children and osteomalacia in adults. An inadequate amount of vitamin D can increase the risk of bone fractures in elderly people and, more recently, it has been associated to an increased risk of cancers, cardiovascular diseases, immunodeficiency, and diabetes.[1,2] Cholecalciferol (vitamin D_3, Figure 1 A) and ergocalciferol (vitamin D_2; Figure 1 B) can be obtained through dietary intake.

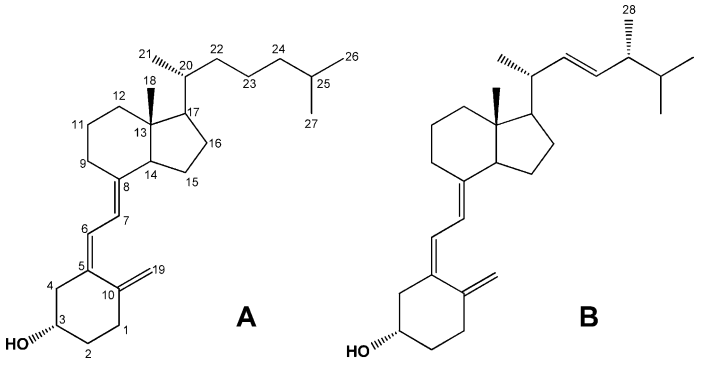

Figure 1. Chemical structures of the two vitamin D forms, cholecalciferol (vitamin D_3, **A**) and ergocalciferol (vitamin D_2, **B**), tested as substrates for the recombinant peroxygenase.

Vitamin D_3, derived from cholesterol metabolism in animals, is naturally present in foods, such as fish, meat, eggs, and milk, whereas vitamin D_2 is present in commercially irradiated mushrooms and yeast,[3] where it is formed from fungal ergosterol.[4] Additionally, vitamin D_3 is produced in human skin and converted to active hydroxylated metabolites in the liver and kidneys, as described in Figure 2. 25-Hydroxyvitamin D_3 (25-hydroxycholecalciferol) is the first metabolically active, and the major form, of circulating vitamin D (used to detect vitamin deficiency), whereas 1α,25-dihydroxyvitamin D_3 (calcitriol) is the major active form. Vitamin D_2 obtained from our diet undergoes an analogous set of activation steps to give 25-hydroxyergocalciferol and 1α,25-dihydroxyergocalciferol.

It has been shown that for diseases, such as hyperglycemia, chronic kidney disease, and for anephric patients, supplementation with 25-hydroxycholecalciferol has a positive effect[5–7] owing to, in some cases, the extrarenal hydroxylation of this metabolite.[6] Similar findings have also been reported for Crohn's disease and intestinal resection, as well as for chronic cholestatic liver disease.[8,9] In addition to the physiological interest in 25-hydroxycholecalciferol for human health, this compound has also raised interest for the feeding of poultry[10–13] and other farm animals.[14] The poor-quality skeletons of broiler chickens is currently a serious problem that has a negative influence on the economic benefits of rearing these birds. The

[a] E. D. Babot, J. C. del Río, Dr. A. Gutiérrez
Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC
Reina Mercedes 10, E-41012 Seville (Spain)
E-mail: anagu@irnase.csic.es

[b] Dr. L. Kalum
Novozymes A/S
Krogshoejvej 36, DK-2880 Bagsvaerd (Denmark)

[c] Prof. A. T. Martínez
Centro de Investigaciones Biológicas, CSIC
Ramiro de Maeztu 9, E-28040 Madrid (Spain)

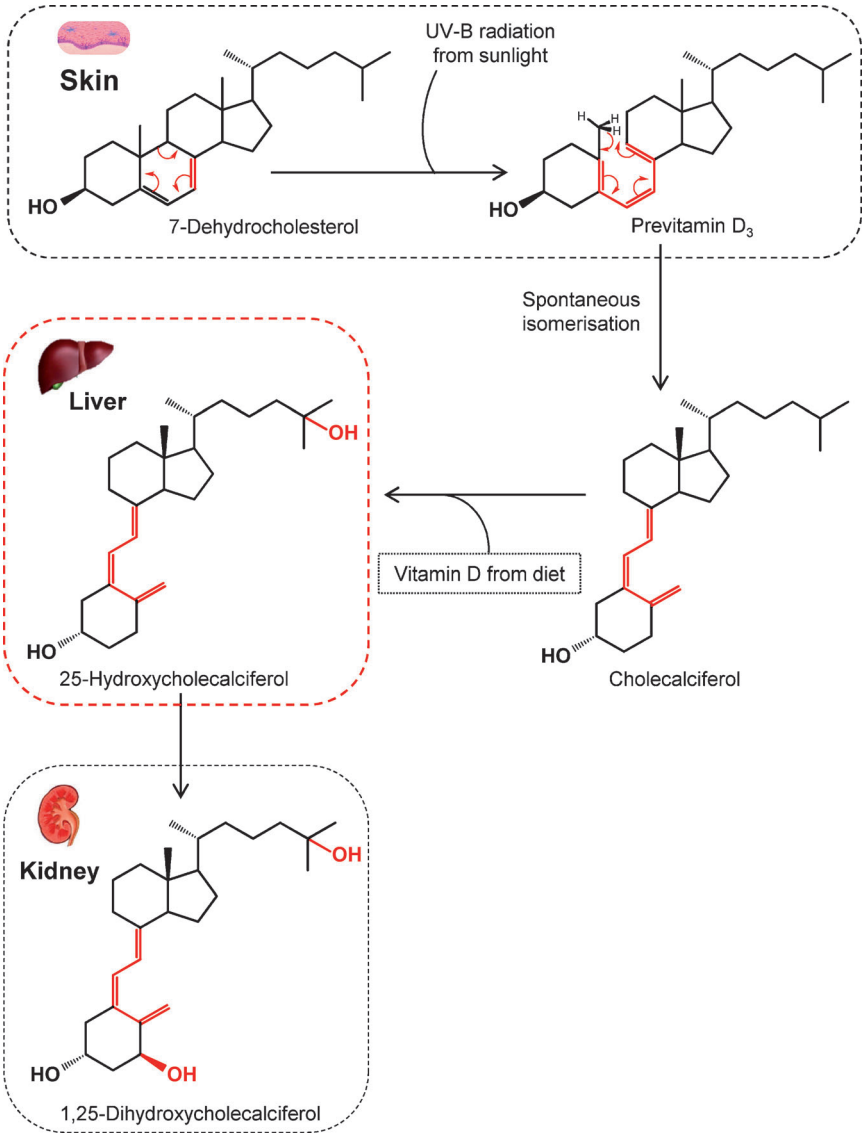

Figure 2. Human metabolism of vitamin D_3 showing the key metabolites. On exposure to UV light in the skin, provitamin D_3 (7-dehydrocholesterol) is converted to previtamin D_3, which is isomerized to more stable vitamin D_3 (cholecalciferol) via a thermally induced transformation. Vitamin D_3, either cutaneously formed or taken in the diet, is then hydroxylated in the liver to 25-hydroxyvitamin D_3 (25-hydroxycholecalciferol). Finally, 25-hydroxyvitamin D_3 is hydroxylated again, primarily in the kidney, to give 1α,25-dihydroxyvitamin D_3 (calcitriol), the major biologically active form of vitamin D.

addition of 25-hydroxycholecalciferol into feed mixtures has been shown to have a favorable effect.[10] Therefore, both vitamin D_3, D_2, and their hydroxylated derivatives are approved food additives.[15]

In addition to the biosynthesis of naturally hydroxylated vitamin D_3 by animals, and production of hydroxylated vitamin D_2 from fungal ergosterol by UV irradiation, chemical synthesis (total or partial) for the production of these active compounds has attracted much interest, and several approaches have been reported.[11,16–22] As a sustainable alternative, biotechnological approaches have been investigated, including microbial oxygenations in the production of vitamin D derivatives.[23,24] Moreover, enzymes (monooxygenases containing both heme and flavin cofactors) are capable of catalyzing the selective oxyfunctionalization of organic substrates under mild and environmentally friendly conditions.[25] Members of the cytochrome P450 monooxygenase (P450) superfamily are remarkable examples of such catalysts.[26–29] However, large-scale applications are limited because of the intrinsic properties of P450s.[30]

Recently, a new type of peroxidase was discovered in the basidiomycete *Agrocybe aegerita*[31] that turned out to be a true peroxygenase efficiently transferring oxygen from peroxide to various organic substrates.[32–34] Only a few similar enzymes have been characterized from related fungi, but there are indications of their widespread occurrence.[35–37] For example, over 100 related genes, classified in the new superfamily of heme–thiolate peroxidases,[33,38] have recently been identified in the screening of 24 sequenced genomes of basidiomycetes,[39] including *Coprinopsis cinerea*. To date, wild peroxygenase has not been isolated from *C. cinerea* cultures, but the corresponding genes from the genome were heterologously expressed by Novozymes A/S (Bagsvaerd, Denmark).[40] The monooxygenation ability of one of these recombinant enzymes (protein ID 7249 from the *C. cinerea* genome available at the JGI; http://genome.jgi.doe.gov/Copci1) has recently been exploited for the hydroxylation of fatty acids, fatty alcohols, and long-chain alkanes.[41] Herein, the ability and efficiency of the recombinant *C. cinerea* peroxygenase in the hydroxylation of vitamin D_3 and D_2, is evaluated for the first time.

Results

The current work proposes a biotechnological approach to obtain both 25-hydroxycholecalciferol and 25-hydroxyergocalciferol starting from readily available and low-cost cholecalciferol and ergocalciferol, respectively, by using an enzyme from *C. cinerea*, which is representative of the new fungal peroxidases with monooxygenase activity (peroxygenases) first described in *A. aegerita*. With this purpose, the reaction of the

latter secosterols with the recombinant peroxygenase was studied, and the substrate conversion rate and product identification were carried out by GC-MS.

Enzymatic hydroxylation of cholecalciferol at the C-25 position

Firstly, cholecalciferol (Figure 1 A) was tested as a peroxygenase substrate. The GC-MS analysis of the reaction revealed that this compound was completely transformed by the *C. cinerea* enzyme within 60 min (Figure 3 A). The control reaction without peroxygenase is shown in Figure 3 B. The conversion showed strict regioselectivity and provided 25-hydroxycholecalciferol exclusively (100 % yield), as confirmed by comparison to a true standard of this compound (Figure 3 C), which showed an identical retention time.

The double peaks observed in the chromatograms for each substrate and product (Figure 3) correspond to the isopyro (19β, 9β) and pyro (19α, 9α) isomers formed by thermal rearrangement involving ring-B closure, which vitamin D and its hydroxylated derivatives undergo because of the temperature at which GC-MS EI(+) analysis is carried out. Indeed, the presence of the two isomers during GC separation is a useful indication that a secosteroid of the vitamin D-type was injected into the system.[42] Therefore, both the chromatographic profiles (Figure 3) and the mass spectra discussed below (Figure 4) correspond to the isomerized secosteroids.

The position of the hydroxyl group at C-25 was established by mass spectrometry of the trimethylsilyl (TMS) derivative. The spectrum in Figure 4 A shows a prominent ion from C-24/C-25 bond cleavage, with a characteristic fragment at *m/z* 131 and a molecular ion at *m/z* 544. Additionally, characteristic fragments at *m/z* 529 ($[M-15]^+$), *m/z* 454 ($[M-90]^+$), *m/z* 439 ($[M-90-15]^+$), *m/z* 349 ($[M-90-90-15]^+$), and *m/z* 413, were also present. The spectrum of standard 25-hydroxycholecalciferol is shown in the inset of Figure 4 A, and is identical to that obtained from the peroxygenase reaction. It should be noted that the mass spectra in Figure 4 are from vitamin D pyroderivatives in which ring-B is cyclized, even if the secosteroid is illustrated, because GC-MS spectra of non-isomerized vitamin D have not yet been described.[42] The NMR spectra of the reaction product confirmed the formation of 25-hydroxylated vitamin D_3 (unprotonated C-25 with $\delta_C = 71.3$ ppm, and other signals listed in the Experimental Section).

An ^{18}O-labeling study, using cholecalciferol as substrate and $H_2{}^{18}O_2$ as enzyme co-substrate, was performed to investigate the origin of the oxygen atom incorporated during oxygenation. The results showed that oxygen originating from ^{18}O-labeled peroxide ($H_2{}^{18}O_2$) was completely incorporated into 25-hydroxycholecalciferol. Mass spectral analysis of the resulting monohydroxylated cholecalciferol (Figure 4 B) showed that the characteristic fragments had approximately 90 % shifted from the natural abundance at *m/z* 544, *m/z* 529, *m/z* 454, *m/z* 439, *m/z* 349, *m/z* 131, and *m/z* 413 found in the unlabeled peroxide reaction to *m/z* 546, *m/z* 531, *m/z* 456, *m/z* 441, *m/z* 351, *m/z* 133, and *m/z* 415, respectively (≈10 % of the original fragments remained in the $H_2{}^{18}O_2$ reactions owing to the 90 % ^{18}O isotopic purity of the labeled peroxide).

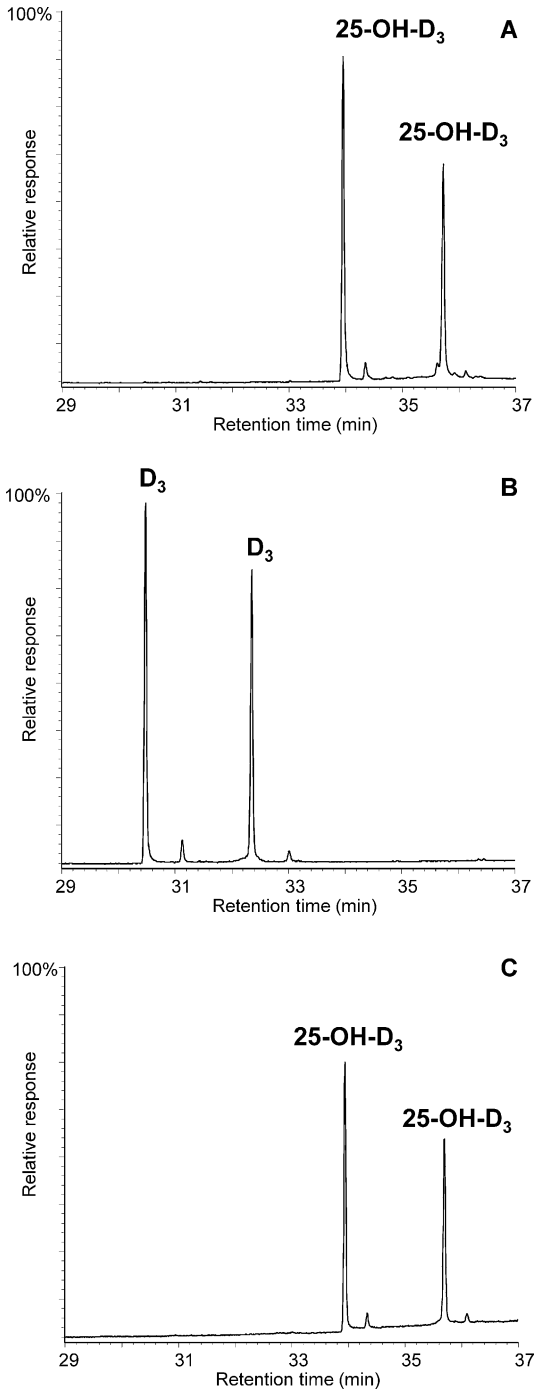

Figure 3. GC-MS analysis of the *C. cinerea* peroxygenase reaction (at 60 min) with cholecalciferol (see Figure 1 A) A) showing product formation, B) control without enzyme, and C) 25-hydroxycholecalciferol standard, as TMS derivatives. In all cases the isopyro (left) and pyro (right) isomers from secosteroid thermal rearrangement are obtained.

Enzymatic hydroxylation of ergocalciferol at the C-25 position

Hydroxylation of ergocalciferol (Figure 1 B), the artificial form of vitamin D derived from UV irradiation of ergosterol, was also

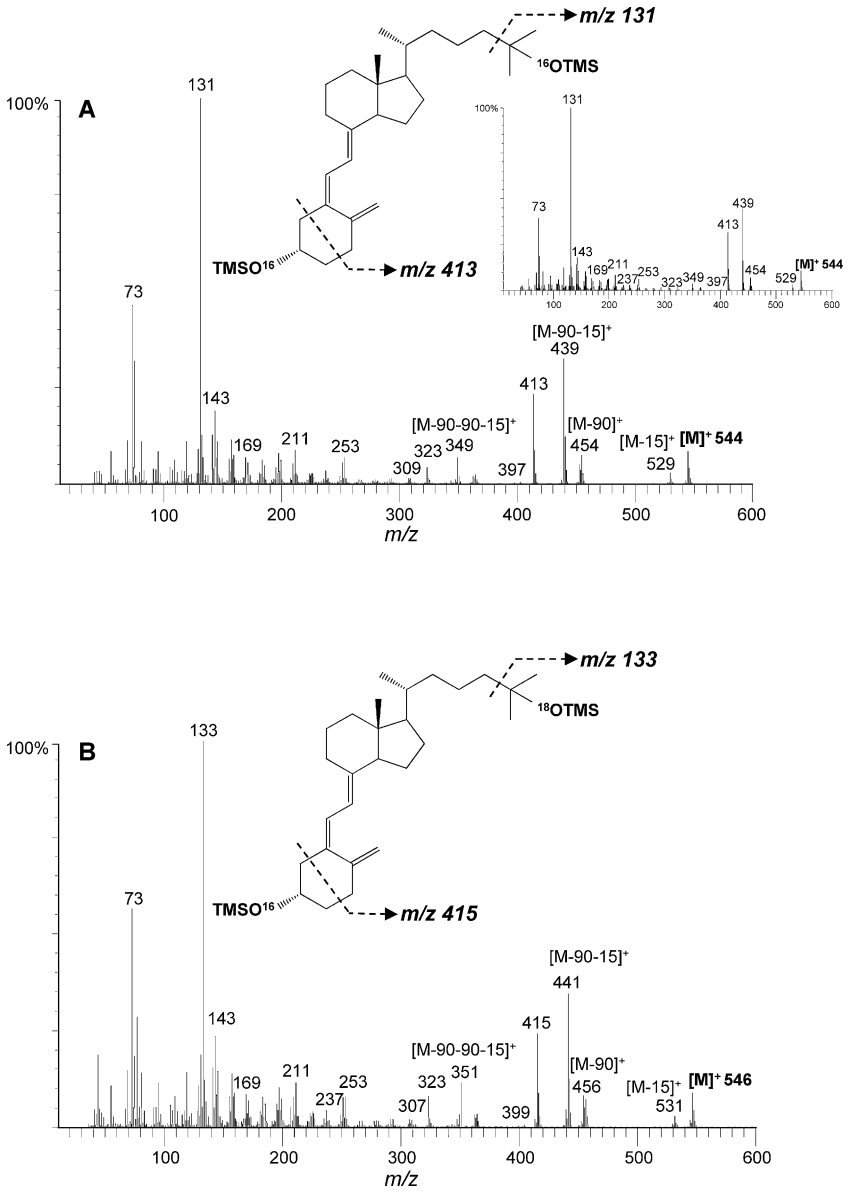

Figure 4. Mass spectra of 25-hydroxycholecalciferol from *C. cinerea* peroxygenase reaction with cholecalciferol in the presence of A) $H_2{}^{16}O_2$ (inset is the mass spectrum of standard compound 25-hydroxycholecalciferol) and B) $H_2{}^{18}O_2$ (90% isotopic purity), as TMS derivatives.

studied. The peroxygenase from *C. cinerea* showed similar efficiency, in the transformation of ergocalciferol, to that of cholecalciferol after 60 min incubation, resulting in 90% substrate conversion (Figure 5 A and B) although the conversion was slower as found at shorter incubation times (data not shown). This reaction also showed strict regioselectivity, 25-hydroxyergocalciferol being the only product formed (90% yield), confirmed by comparison with a standard (Figure 5 C).

The position of the hydroxyl group at C-25 was established from the mass spectrum (Figure 6), which was identical to that of a standard (Figure 6, inset). The spectrum shows a prominent ion from C-24/C-25 bond cleavage, with a characteristic fragment at *m/z* 131 and a molecular ion at *m/z* 556. Additionally, characteristic fragments at *m/z* 541 ($[M-15]^+$), *m/z* 466 ($[M-90]^+$), *m/z* 451 ($[M-90-15]^+$), and *m/z* 361 ($[M-90-90-15]^+$), were also present in the spectrum. In a similar manner to the reaction with cholecalciferol, the peaks of the two pyroisomers are found in the chromatogram (Figure 5), and the mass spectra in Figure 6 are from isomerized vitamin D derivatives. The NMR spectra of the reaction product confirmed the formation of 25-hydroxylated vitamin D_2 (unprotonated C-25 with $\delta_C = 72.5$ ppm, and other signals listed in the Experimental Section).

Similar to the cholecalciferol reaction, ^{18}O-labeling showed that oxygen originating from ^{18}O-labeled peroxide ($H_2{}^{18}O_2$) was completely incorporated into 25-hydroxyergocalciferol. Mass spectral analysis (Figure 6 B) showed that the characteristic fragments for the 25-hydroxyergocalciferol had approximately 90% shifted from the natural abundance at *m/z* 556, *m/z* 541, *m/z* 466, *m/z* 451, *m/z* 361, and *m/z* 131 found in the unlabeled peroxide reaction to *m/z* 558, *m/z* 543, *m/z* 468, *m/z* 441, *m/z* 363, and *m/z* 133, respectively.

Discussion

It has been demonstrated that the 25-hydroxylated metabolites of vitamin D, including 25-hydroxycholecalciferol and 25-hydroxyergocalciferol, are considerably better therapeutic agents for several diseases than vitamin D itself, owing to their direct biological activity and better intestinal absorption in some cases.[5,8,9] Likewise, these hydroxylated compounds also find applications as animal-feed supplements (and are superior to vitamin D itself), especially when their subsequent metabolic conversion is diminished.[10,43,44] On the other hand, despite some controversy on which oral formulation of vitamin D (vitamin D_3 or D_2) is preferred, and although there are reports that certain animal species discriminate against vitamin D_2, in humans vitamin D_2 and D_3 seem to be essentially equipotent in the prevention and cure of rickets and possibly other vitamin D actions in the body.[6] The recognition that both forms of 25-hydroxyvitamin D exhibit several advantages relative to vitamin D has gen-

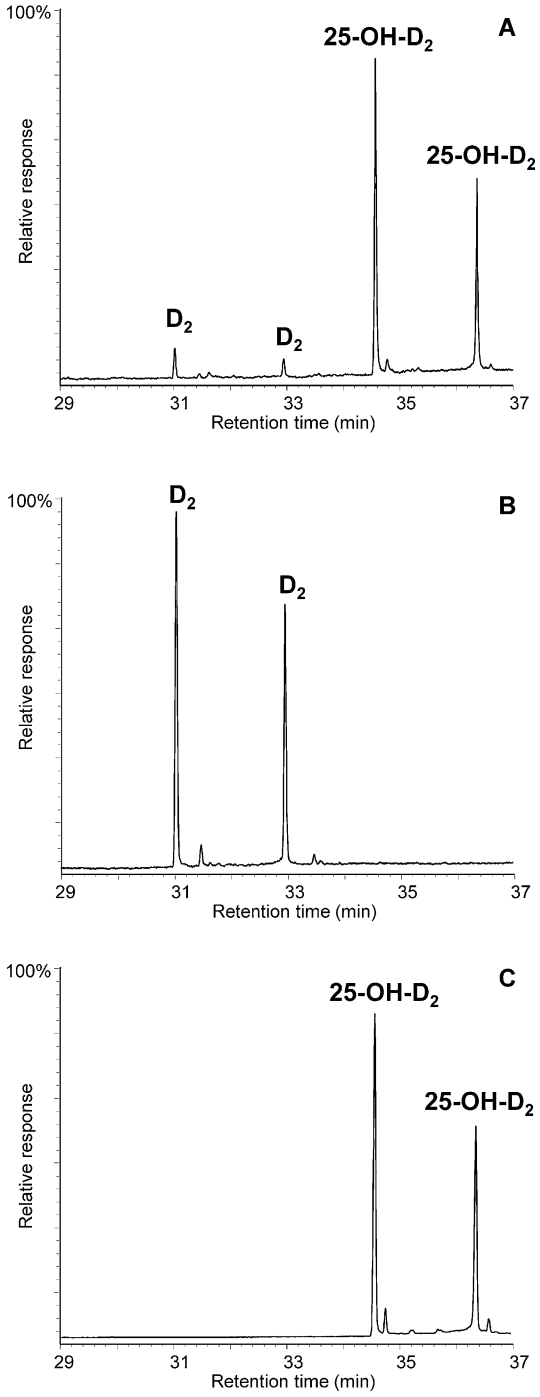

Figure 5. GC-MS analysis of the *C. cinerea* peroxygenase reaction (at 60 min) with ergocalciferol (see Figure 1 B) A) showing product formation, B) control without enzyme, and C) 25-hydroxyergocalciferol standard, as TMS derivatives. In all cases the isopyro (left) and pyro (right) isomers from secosteroid thermal rearrangement are obtained.

erated great interest in the chemical synthesis of these compounds.[11,16–20] However, these reactions often require strong oxidizing agents, include several steps, are expensive, and occur with little regioselectivity in the hydroxylation steps.

In contrast to chemical synthesis, the peroxygenase from the *C. cinerea* genome is highly selective, and can hydroxylate the C-25 position of both vitamin D_3 and D_2 to provide the biologically active hydroxylated forms by means of environmentally friendly and potentially unexpensive reactions. This enzyme, the sequence of which is also available from GenBank (XM_001831858), shares 63% of mature protein sequence with the first described and cloned basidiomycete peroxygenase from *A. aegerita*. Similarities include the catalytically relevant Cys36 (87% identity in the Lys15–Gly66 region around this heme-iron ligand), Arg189 and Glu196 (involved in reactions with peroxide), as well as four (Phe76, Phe121, Phe191, and Phe199) of the five phenylalanine residues at the heme access cavity of the *A. aegerita* peroxygenase, and two cysteine residues (Cys284 and Cys327).[45] These structural similarities justify the similar oxygenation activities reported in a recent comparison of two basidiomycete peroxygenases.[41] However, the *C. cinerea* enzyme is superior for vitamin D_2/D_3 hydroxylation, resulting in the 90–100% conversion rates reported herein, whereas 45–77% conversion was obtained with the *A. aegerita* and *Marasmius rotula* peroxygenases under the same reaction conditions (unpublished).

Selective hydroxylation of vitamin D by the recombinant *C. cinerea* peroxygenase, as shown here by MS and NMR analysis, is in agreement with the described subterminal oxygenation of fatty acids, fatty alcohols, and alkanes by this enzyme,[41] as well as by the *A. aegerita* peroxygenase.[46,47] It is worth noting that the structural features of the vitamin D_2 side chain, such as the methyl group at C-24 and the double bond at C-22, did not preclude the hydroxylation at C-25. In the above reactions, the basidiomycete enzymes act as true peroxygenases, as confirmed by the labeled oxygen of $H_2{}^{18}O_2$ being incorporated into the formed products. The same was shown previously, for the peroxygenase-catalyzed oxygenation of several aliphatic[46] and aromatic compounds,[34] in a series of reactions that could also be useful for preparing specifically labeled molecules for diagnostic purposes. The enzymatic reactions described herein could be used for the selective hydroxylation of totally or partially chemically synthesized cholecalciferol (or ergocalciferol) or, more interestingly, could be applied to natural ergocalciferol obtained by UV irradiation of ergosterol, a sterol characteristic of fungi.[48] The latter UV reaction is possible because the ergosterol ring-B is doubly unsaturated, compared with the monounsaturated ring in the main sterols of plant (sitosterol) and animal (cholesterol) origin (7-dehydrocholesterol is a precursor of both cholesterol and vitamin D_3).

The chemistry of aliphatic- (and aromatic-) substrate oxygenation by the basidiomycete peroxygenases[49,50] is the same as that operating in P450s,[27] both enzymes share a heme–thiolate active center and a Fe^{IV}=O compound that transfers the oxygen atom to the substrate in a two-electron oxidation. However, unlike P450s, which are intracellular enzymes whose activation requires NAD(P)H as an electron donor and auxiliary flavin reductases (or a second flavin domain) for electron transfer to O_2 (as shown using $^{18}O_2$),[51] the basidiomycete peroxygenases are secreted proteins, therefore, are far more stable, and only require H_2O_2 for (direct) activation and subsequent oxygen transfer to the substrate.[34,52] Owing to the above characteristics, these new basidiomycete enzymes can be considered as the biocatalysts of choice for enzymatic oxygenations.

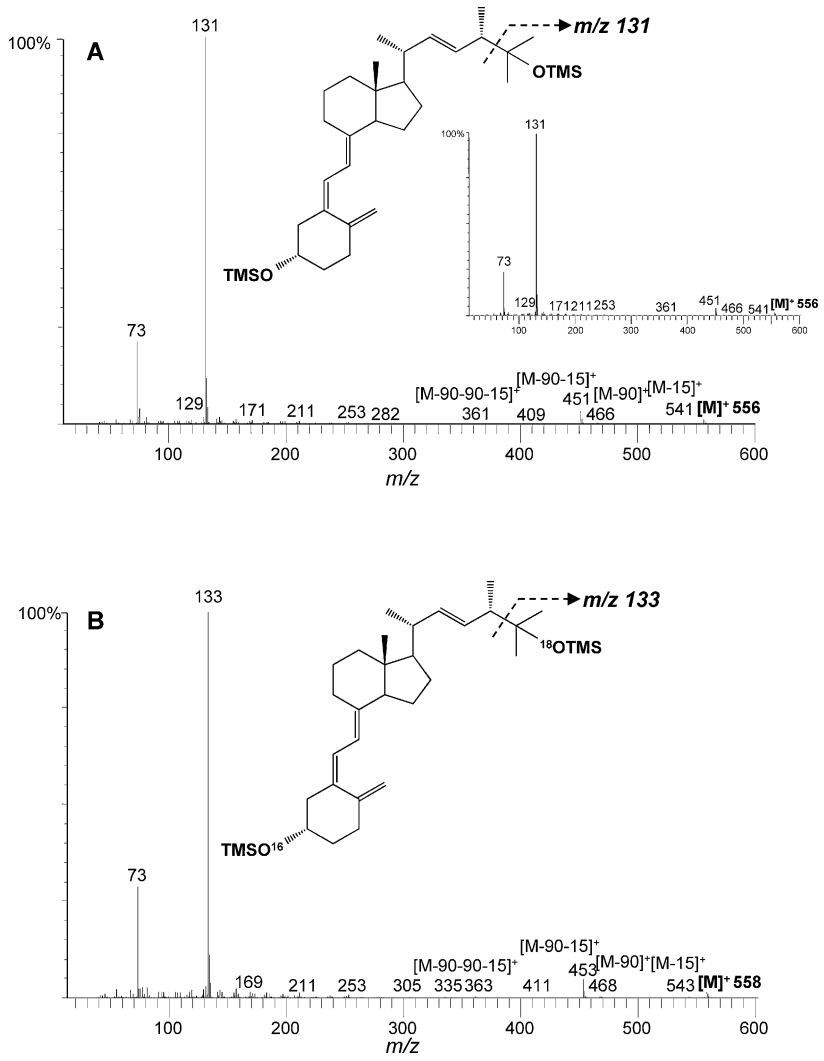

Figure 6. Mass spectra of 25-hydroxyergocalciferol from *C. cinerea* peroxygenase reaction with ergocalciferol in the presence of A) $H_2{}^{16}O_2$ (inset is the mass spectrum of standard compound 25-hydroxyergocalciferol) and B) $H_2{}^{18}O_2$ (90% isotopic purity), as TMS derivatives.

The above reasoning is especially true for the vitamin D hydroxylation reactions described above because, in addition to their chemical selectivity, high conversion rates were obtained resulting in almost complete transformation in a short reaction time. The efficiency of these peroxygenase reactions is similar to that recently found for the oxygenation of some alkylbenzenes and styrene derivatives,[53] but is much higher than that reported for most peroxygenase reactions, such as fatty acid or alkane oxygenations, which often result in several products (at ω-1 and ω-2 positions) with different oxidation degrees (from hydroxyl to carbonyl groups).[41,46,47] Therefore, the production of biologically active vitamin D forms from both cholecalciferol and ergocalciferol adds to the list of interesting oxygenation reactions that basidiomycete peroxygenase can catalyze,[46,47,54–58] occupying a preeminent position owing to the high degree of regioselectivity of the enzymatic bioconversion. Moreover, the recombinant nature of the *C. cinerea* peroxygenase used, after successful expression in the industrial host *Aspergillus oryzae*,[40] permits over expression of the enzyme (as a low-cost product) and tailoring of its properties by protein engineering tools, if necessary, to improve industrial applicability.

Conclusions

Fungal peroxygenase, a novel biocatalyst for environmentally friendly oxyfunctionalization of a variety of aromatic and aliphatic compounds, first described in *A. aegerita*, is now available for application in enzymatic reactions after the heterologous expression of a *C. cinerea* peroxygenase gene in an industrial host organism. The recombinant enzyme obtained is evaluated for the first time in the hydroxylation of vitamin D, including both the cholecalciferol and ergocalciferol forms. The 100% regioselectivity in the hydroxylation at the C-25 position, together with its self-sufficient oxygenase activity (i.e. the ability to catalyze oxygenations without the help of intracellular enzymes providing electrons and reducing power, as in the case of P450s), and its overexpression in a suitable host organism, make this recombinant heme–thiolate peroxidase an interesting industrial biocatalyst for the synthesis of 25-hydroxycalciferol.

Experimental Section

Enzyme

The recombinant peroxygenase used in this study corresponds to gene model CC1G_08427T0 from the sequenced *C. cinerea* genome available at the JGI (http://genome.jgi.doe.gov/copci1) and GenBank (XM_001831858). The corresponding protein (*C. cinerea* genome ID 7249) was produced by heterologous expression by using a Novozymes *Aspergillus oryzae* industrial host system,[40] and purified by using a combination of S-Sepharose and SP-Sepharose ion-exchange chromatography. The recombinant peroxygenase preparation is an electrophoretically homogeneous glycoprotein with a molecular mass around 44 kDa (a non-uniform glycosylation pattern was observed), a typical UV/Vis spectrum with a Soret band at 418 nm, and the ability to oxygenate different aromatic compounds[59] with a specific activity of approximately 100 U mg^{-1} measured with veratryl alcohol. One enzyme activity unit was defined as the amount of enzyme required to oxidize 1 μmol of veratryl alcohol to veratraldehyde (ε_{310} 9300 M^{-1} cm^{-1}) in 1 min at 24 °C, pH 7, in the presence of 0.5 mM H_2O_2.

Secosteroid substrates and standards

Vitamin D_3, also known as calciol or cholecalciferol ((5*Z*,7*E*)-(3*S*)-9,10-seco-5,7,10(19)-cholestatrien-3-ol), and vitamin D_2, also known as ercalciol or ergocalciferol ((5*Z*,7*E*,22*E*)-(3*S*)-9,10-seco-5,7,10(19),22-ergostatetraen-3-ol), were tested as substrates of the *C. cinerea* peroxygenase. 25-Hydroxyvitamin D_3, also known as calcidiol or 25-hydroxycholecalciferol ((5*Z*,7*E*)-(3*S*)-9,10-seco-5,7,10(19)-cholestatriene-3,25-diol), and 25-hydroxyvitamin D_2, also known as ercalcidiol or 25-hydroxyergocalciferol ((5*Z*,7*E*,22*E*)-(3*S*)-9,10-seco-5,7,10(19),22-ergostatetraene-3,25-diol), were used as standards for GC-MS analyses. All the compounds were from Sigma–Aldrich.

Enzymatic reactions

Reactions of cholecalciferol and ergocalciferol (0.05 mM) with the *C. cinerea* peroxygenase (1 U) were performed in 5 mL of 50 mM sodium phosphate (pH 7) at 40 °C for 60 min, in the presence of 0.5 mM H_2O_2. The substrates were previously dissolved in acetone, and added to the buffer (the acetone concentration in the reaction was 40%). In the control experiments, the substrates were treated under the same conditions (including 0.5 mM H_2O_2), but in the absence of the enzyme. Enzymatic reactions with ^{18}O-labeled hydrogen peroxide [$H_2{}^{18}O_2$, 90% isotopic content, Sigma–Aldrich (2% w/v solution)] were also performed under the same conditions as described above.

After the enzymatic reactions, products were recovered by liquid–liquid extraction with methyl *tert*-butyl ether, dried under N_2, and redissolved in chloroform for GC-MS analysis. Bis(trimethylsilyl)trifluoroacetamide (Supelco) in the presence of pyridine was used to prepare TMS derivatives. An internal standard was added after the enzymatic reactions to determine product yields.

GC-MS analyses

The GC-MS analyses were performed with a Shimadzu GC-MS QP2010 Ultra, using a fused-silica DB-5HT capillary column (30 m × 0.25 mm internal diameter, 0.1 μm film thickness) from J&W Scientific.[60] The oven was heated from 120 °C (1 min) to 300 °C (15 min) at 5 °C·min^{-1}. The injection was performed at 300 °C, the transfer line was kept at 300 °C, and helium was used as carrier gas.

Compounds were identified by mass fragmentography, and by comparison of the mass spectra with those of the Wiley and NIST libraries and standards, and quantitation was obtained from total-ion peak area by using the response factors of the same reaction products (25-hydroxycholecalciferol and 25-hydroxyergocalciferol). These two standards were also used as external standards for calculation of product yields. The relative abundance of the hydroxylated product incorporating one $^{18}O_2$ atom in the $H_2{}^{18}O_2$ reactions described above was estimated by peak integration using the corresponding ion with a 2 *m/z* increase (with correction from interfering ions in $H_2{}^{16}O_2$ spectra, when required).

NMR analyses

The structure of the 25-hydroxy products was confirmed by ^{1}H, ^{13}C, and HSQC NMR spectroscopy (the latter enabling assignment in the 1D spectra). Spectra of both the products and the standards were acquired. The NMR spectra were acquired on a Bruker Biospin (Billerica, MA) AVANCE 500 MHz spectrometer fitted with a cryogenically cooled 5 mm TCI gradient probe with inverse geometry (proton coils closest to the sample).

25-Hydroxycholecalciferol: ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 0.55$ (3H, s, 18-H), 0.94 (1H, d, $J = 6.5$ Hz, 21-H), 1.06 (1H, m, 22-H), 1.22 (3H, s, 26-H), 1.22 (3H, s, 27-H), 1.23 (1H, m, 23-H), 1.27 (1H, m, 16-H), 1.28 (1H, m, 14-H), 1.29 (1H, m, 12-H), 1.37 (1H, m, 22-H), 1.38 (1H, m, 20-H), 1.39 (1H, m, 24-H), 1.42 (1H, m, 23-H), 1.44 (1H, m, 24-H), 1.47 (2H, m, 11-H), 1.53 (1H, m, 15-H), 1.66 (1H, m, 15-H), 1.67 (1H, m, 2-H), 1.67 (1H, m, 9-H), 1.87 (1H, m, 16-H), 1.92 (1H, m, 2-H), 1.98 (1H, m, 17-H), 2.06 (1H, m, 12-H), 2.17 (1H, m, 1-H), 2.40 (1H, m, 1-H), 2.57 (1H, dd, $J = 3.7$, 13.1 Hz, 4-H), 2.82 (1H, m, 9-H), 3.95 (1H, bm, 3-H), 4.82 (1H, m, 19-H), 5.05 (1H, m, 19-H), 6.03 (1H, d, $J = 11.2$ Hz, 7-H), 6.23 ppm (1H, d, $J = 11.2$ Hz, 6-H); ^{13}C NMR (500 MHz, $CDCl_3$): $\delta = 12.2$ (C-18), 19.0 (C-21), 21.0 (C-23), 22.4 (C-11), 23.7 (C-15), 27.8 (C-16), 29.2 (C-9), 29.4 (C-27), 29.5 (C-26), 32.1 (C-1), 35.3 (C-2), 36.3 (C-20), 36.6 (C-22), 40.7 (C-12), 44.6 (C-24), 46.0 (C-13), 46.1 (C-4), 56.5 (C-17), 56.7 (C-14), 69.4 (C-3), 71.3 (C-25), 112.6 (C-19), 117.7 (C-7), 122.2 (C-6), 135.2 (C-5), 142.4 (C-8), 145.3 ppm (C-10).

25-Hydroxyergocalciferol: ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 0.56$ (3H, s, 18-H), 1.00 ppm (3H, d, $J = 6.9$ Hz, 28-H), 1.04 (3H, d, $J = 6.7$ Hz, 21-H), 1.13 (3H, s, 26-H), 1.17 (3H, s, 27-H), 1.26 (1H, m, 16-H), 1.31 (1H, m, 12-H), 1.34 (1H, m, 14-H), 1.46 (2H, m, 11-H), 1.54 (1H, m, 15-H), 1.68 (1H, m, 15-H), 1.68 (1H, m, 2-H), 1.68 (1H, m, 9-H), 1.71 (1H, m, 16-H), 1.92 (1H, m, 2-H), 1.96 (1H, m, 12-H), 1.99 (1H, m, 17-H), 2.07 (1H, m, 20-H), 2.11 (1H, m, 24-H), 2.18 (1H, m, 1-H), 2.29 (1H, dd, $J = 13.0$, 7.3 Hz, 4-H), 2.40 (1H, m, 1-H), 2.57 (1H, dd, $J = 13.0$, 3.7 Hz, 4-H), 2.83 (1H, m, 9-H), 3.95 (1H, m, 3-H), 4.81 (1H, m, 19-H), 5.04 (1H, m, 19-H), 5.30 (1H, dd, $J = 15.3$, 8.2 Hz, 23-H), 5.37 (1H, dd, $J = 15.3$, 8.3 Hz, 22-H), 6.03 (1H, d, $J = 11.3$ Hz, 7-H), 6.23 (1H, d, $J = 11.3$ Hz, 6-H); ^{13}C NMR (500 MHz, $CDCl_3$): $\delta = 12.4$ (C-18), 15.8 (C-28), 21.1 (C-21), 22.4 (C-11), 23.7 (C-15), 26.5 (C-26), 27.2 (C-27), 27.9 (C-16), 29.1 (C-9), 32.1 (C-1), 35.3 (C-2), 40.6 (C-12), 40.6 (C-20), 46.0 (C-13), 46.1 (C-4), 48.3 (C-24), 56.4 (C-14), 56.5 (C-17), 69.3 (C-3), 72.5 (C-25), 112.6 (C-19), 117.7 (C-7), 122.6 (C-6), 129.3 (C-23), 135.3 (C-5), 139.2 (C-22), 142.2 (C-8), 145.2 ppm (C-10).

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