# Supporting Information

## Molecular determinants for selective $C_{25}$-hydroxylation of vitamins $D_2$ and $D_3$ by fungal peroxygenases

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The Supporting Information shows the main interactions of cholecalciferol and ergocalciferol at the peroxygenase heme access channel (**Fig. S1** and **S2**, respectively), the effect of side-chain structure on the conversion rates of five sterols by the *A. aegerita* and *C. cinerea* peroxygenases (**Table S1**), and distributions of estimated Fe=O-H and O-H-C angles in the peroxygenase reactions with cholecalciferol and ergocalciferol (**Fig. S3**).

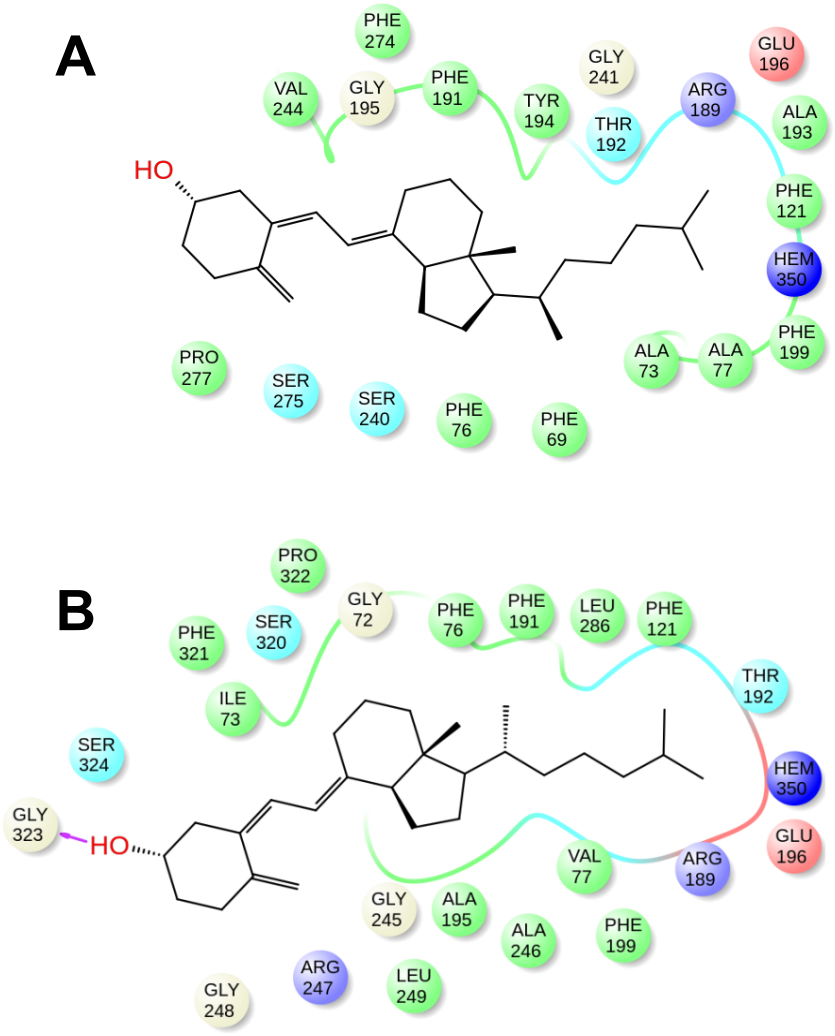



**Fig. S1** Main interactions (below 3 Å) for cholecalciferol with the peroxygenases of *A. aegerita* (**A**) and *C. cinerea* (**B**).

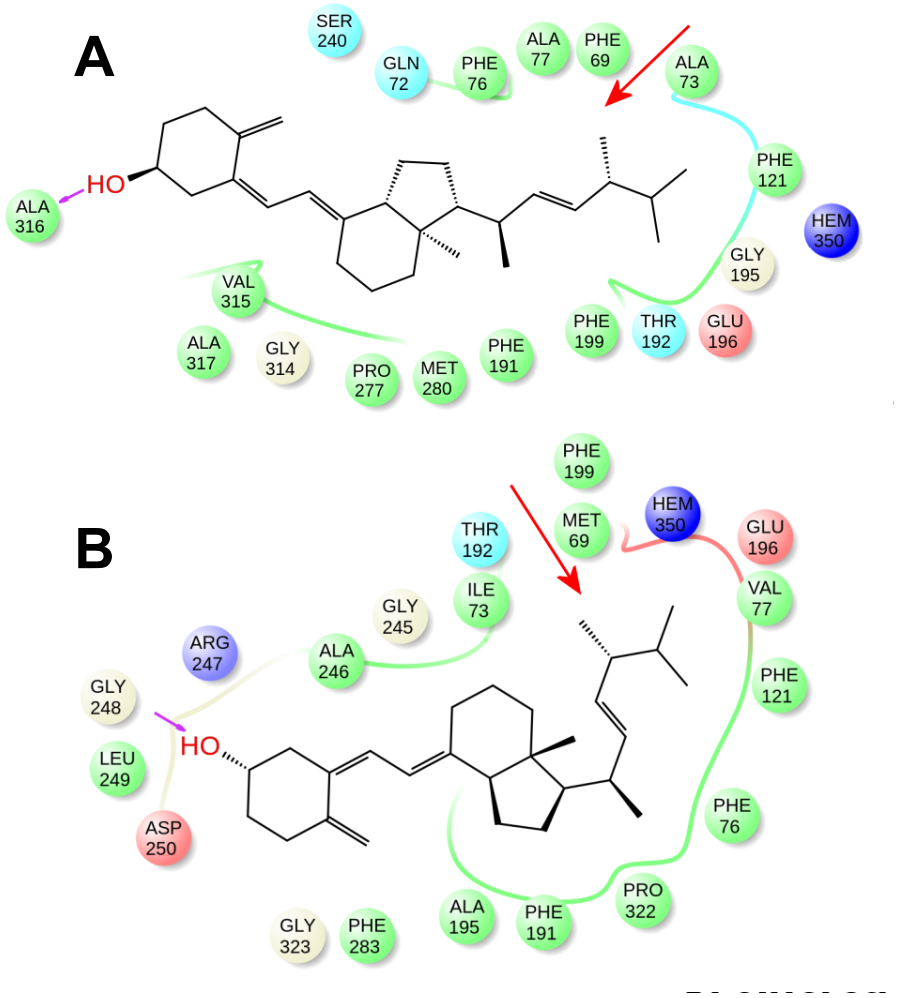
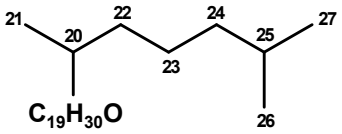
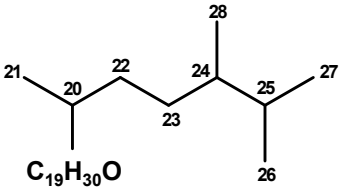
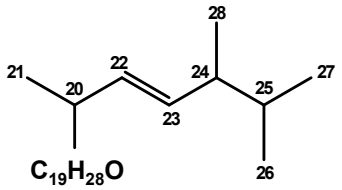
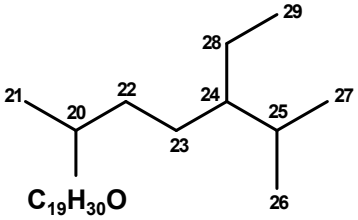
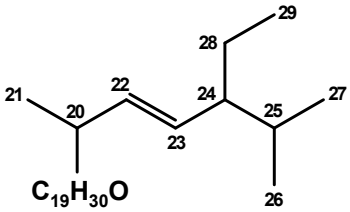



**Fig. S2** Main interactions (below 3 Å) for ergocalciferol with the peroxygenases of *A. aegerita* (**A**) and *C. cinerea* (**B**).

**Table S1** Conversion degree of steroids with different side-chains (*A*-*E*: cholesterol, campesterol, ergosterol, sitosterol and stigmasterol, respectively) by *A. aegerita* and *C. cinerea* peroxygenases[a]

| | | *Conversion (%)* | |
|---|---|---|---|
| | | *A. aegerita* peroxygenase | *C. cinerea* peroxygenase |
| *A* |   | 64 | 100 |
| *B* |   | 47 | 30 |
| *C* |   | 10 | 6 |
| *D* |   | 13 | 6 |
| *E* |   | 2 | 2 |

[a]From E. D. Babot, J. C. del Río, M. Cañellas, F. Sancho, F. Lucas, V. Guallar, L. Kalum, H. Lund, G. Gröbe, K. Scheibner, R. Ullrich, M. Hofrichter, A. T. Martínez, and A. Gutiérrez. 2015. Steroid hydroxylation by basidiomycete peroxygenases: A combined experimental and computational study. *Appl. Environ. Microbiol.* 81:4130-4142

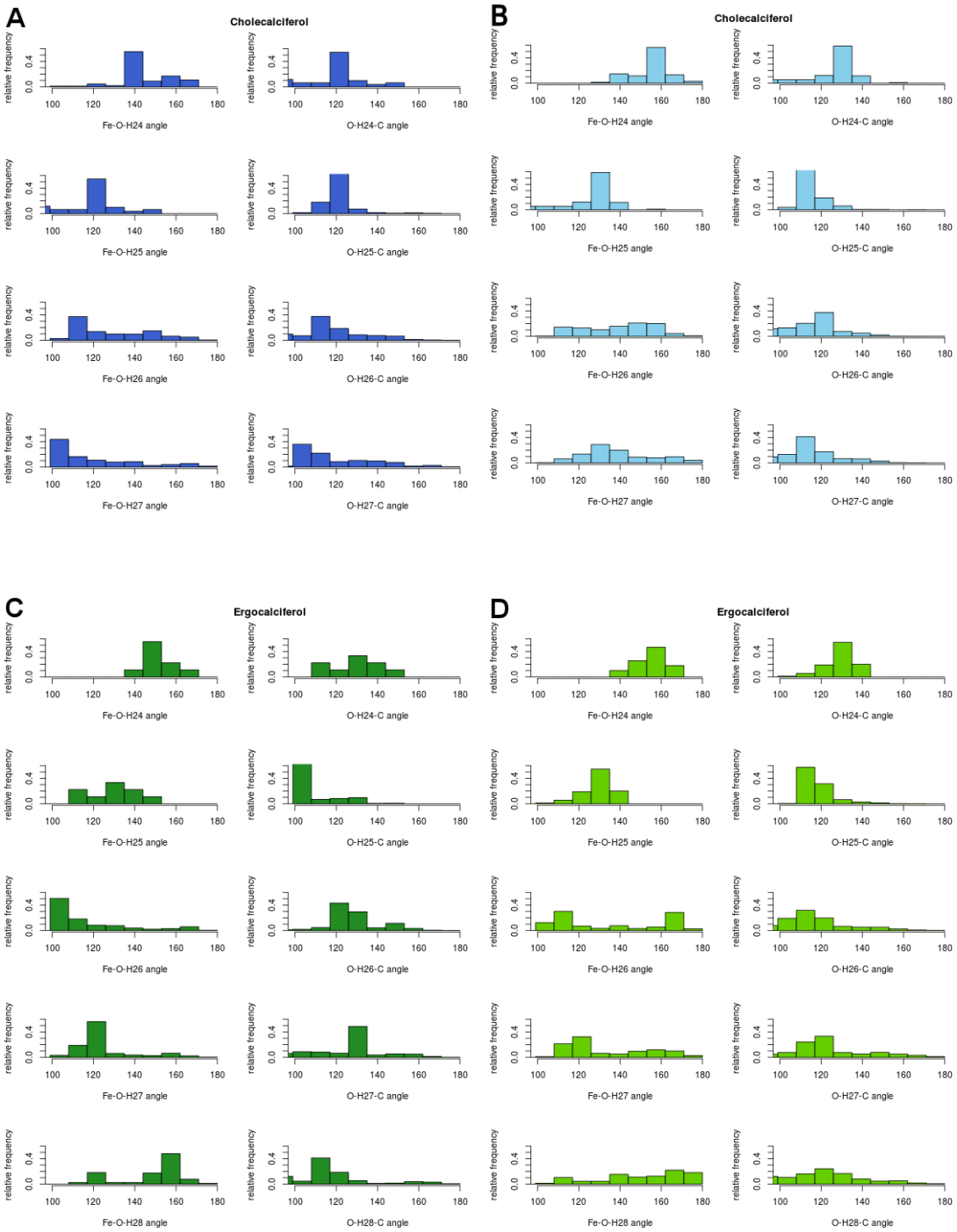



**Fig. S3** Relative distributions of computed Fe=O-H and O-H-C angles for the reactions of *A. aegerita* (**A**, **C**) and *C. cinerea* (**B**, **D**) peroxygenases at the $C_{24}$, $C_{25}$, $C_{26}$ and $C_{27}$, positions of cholecalciferol (blue) and ergocalciferol (green) and the $C_{28}$ position of ergocalciferol. Structures were filtered by energy and distance between the pertinent hydrogen atom and the haem compound I oxygen (below 3 Å). QM/MM studies for $P450_{cam}$[b] show that in a pre-arranged reactive position camphor adopts angles of 130° for Fe=O-H and 170° for O-H-C. While for that latter no correlation is found to the theoretical predictions, in the case of Fe=O-$H_{24}$ angles between 120 and 140° are favoured in $C_{24}$ for cholecalciferol reacting with *A. aegerita* (**A**) in

agreement with the experimentally observed formation of 21% product in this position. In the case of the remaining 3 reactions, few structures are found with angles below 145$^{o}$. In the case of Fe=O-$H_{25}$ (as expected) all compounds present favourable angles. Analogous to the case of the distance distribution analysis, no conclusions can be made for the reactivity of $C_{26}$, $C_{27}$ and $C_{28}$.

[b]J. C. Schöneboom, S. Cohen, H. Lin, S. Shaik and W. Thiel, J. Am. Chem. Soc., 2004, 126, 4017-4034