Supplemental Information for

Controlled oxidation of remote $sp^3$ C—H bonds in artemisinin via P450 catalysts with fine-tuned regio- and stereoselectivity

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Table S1. Sequence of the oligonucleotides used for the preparation of the P450 libraries.

| Primer          | Sequence                                                      |
|-----------------|---------------------------------------------------------------|
| 74nnk_for       | 5'-GATAAAAAACTTAAGTCANNKCTTAAATTC-3'                         |
| 74nnk_rev       | 5'-GAATTAAAGMNNTTGAATAGTTTTATC-3'                           |
| 78nnk_for       | 5'-AATTNNKCTGATAGTGTTGGG-3'                                 |
| 78nnk_rev       | 5'-ACACTATACGMNNAATT-3'                                     |
| 81nnk_for       | 5'-CGTGATNNKGTGGAGACGGGTAA-3'                               |
| 81nnk_rev       | 5'-TAACCCGTCTCCACMNATCAGC-3'                                |
| 87nnk_for       | 5'-GACGGGTANTKCAAGCTGACGCATG-3'                             |
| 87nnk_rev       | 5'-CATGCGTCCAGCTTGMNNTAACCAGTC-3'                           |
| 181nnk_for      | 5'-CGTGCANNKATGAAGTAATGAACAAGC-3'                           |
| 181nnk_rev      | 5'-GCTTCATTACCACTCMNNGAAGC-3'                               |
| 184nnk_for      | 5'-GACTGGATGAANNKATGAACAAGC-3'                              |
| 184nnk_rev      | 5'-CTTGTTCATMNNTTATCCAGTC-3'                                |
| 78-87nnk_for    | 5'-AATTNNKCTGATAGTGTTGGAGACGGGTANTKCAAA-3'                  |
| 78-87nnk_rev    | 5'-TTGTMNNTAACCAGTCCAACTACATACGMNNAATT-3'                   |
| 78-81-82nnk_for | 5'-AATTNNKCTGATNNKNKCGAGACGGGTAA-3'                         |
| 78-81-82nnk_rev | 5'-TAACCCGTCTCCMNANATCACGMNNAATT-3'                         |
| 81-82nnk_for    | 5'-CGCACGTGATNNKNKGGAGACGGGTAA-3'                           |
| 81-82nnk_rev    | 5'-TAACCCGTCTCCMNANATCACGTGC-3'                             |
| 181-184nnk_for  | 5'-GTCGGTGACANNKATGAANNKATGAACAAGC-3'                       |
| 181-184nnk_rev  | 5'-CTTGTTCATMNNTCTAMNNTGCACGGAC-3'                          |
Table S2. Data corresponding to the P450 training sets used for calculating the fingerprint-based model for predicting ART reactivity: fingerprint components (= activity on probe P1 to P5), ART turnovers (TTN), and relative ART activity (normalized to parent FL#62). The regio- and stereoselectivity of the variants are also indicated. The main table refers to training set A used for ranking the #FL62-derived variants, whereas training set B (ranking of II-E2-derived variants) included II-E2.

| Variant | Fingerprint components | TTN         | Rel. activity | Product distribution |
|---------|------------------------|-------------|--------------|----------------------|
|         | P1  P2  P3  P4  P5 |             |              |                      |
| FL#62   | 11.875 10.800 5.500 5.000 9.111 | 339 ± 12    | 1.000        | 83% 10% 7%           |
| 0-#48   | 6.625 10.600 3.500 4.600 2.111 | 20 ± 1      | 0.059        | 27% 73% 0%           |
| I-C1    | 6.941 0.727 1.232 0.988 0.983 | 0           | 0.000        | - - -                |
| I-F7    | 0.823 0.496 0.840 0.672 0.871 | 0           | 0.000        | - - -                |
| I-F9    | 0.892 0.495 0.872 0.622 0.872 | 0           | 0.000        | - - -                |
| I-G11   | 0.776 0.473 0.871 0.696 0.866 | 0           | 0.000        | - - -                |
| I-G3    | 2.312 7.194 5.930 4.174 1.250 | 0           | 0.000        | - - -                |
| II-C1   | 1.303 0.411 3.766 0.696 1.090 | 0           | 0.000        | - - -                |
| II-D10  | 0.572 0.236 0.737 0.592 0.885 | 0           | 0.000        | - - -                |
| II-G10  | 0.546 0.241 0.778 0.479 0.712 | 0           | 0.000        | - - -                |
| II-H4   | 0.519 0.209 0.620 0.433 0.941 | 0           | 0.000        | - - -                |
| III-A3  | 3.093 3.745 1.611 5.910 1.456 | 90 ± 13     | 0.267        | 70% 27% 3%           |
| III-A4  | 0.670 0.322 0.791 0.549 0.930 | 0           | 0.000        | - - -                |
| III-B9  | 1.041 0.703 2.480 0.990 1.194 | 29 ± 2      | 0.086        | 0% 63% 37%           |
| III-C7  | 2.791 3.071 1.915 4.736 1.630 | 106 ± 11    | 0.313        | 39% 59% 2%           |
| III-C8  | 0.533 0.331 1.063 0.518 0.953 | 0           | 0.000        | - - -                |
| III-C9  | 0.509 0.277 0.870 0.465 0.967 | 0           | 0.000        | - - -                |
| III-E3  | 0.637 0.396 1.137 0.494 0.965 | 0           | 0.000        | - - -                |
| III-G3  | 0.775 0.300 0.870 0.563 0.870 | 0           | 0.000        | - - -                |
| III-H9  | 0.690 0.297 0.798 0.545 0.935 | 0           | 0.000        | - - -                |
| II-E2   | 3.626 3.144 1.172 6.469 1.267 | 393 ± 25    | 1.159        | 22 30 48             |
Table S3. Catalytic turnovers, regioselectivity, and stereoselectivity in artemisinin oxidation of the 50 top-scoring FL#62-derived P450 variants identified via fingerprint-based predictions. FL#62 is included for comparison. Stereoselectivity for C7-hydroxylation (expressed as diastereomeric excess, de) is indicated when higher than 60%. Variants are grouped according to their site-selectivity and highlighted as follows: red: variants with >85% 7(S)-selectivity; green: variants with >50% 7(R)-selectivity; blue: variants with >30% 6a-selectivity. Variants highlighted in darker colors correspond to those selected for further characterization (Table 1 in main text).

| Variant | Product distribution | % de (C7) | TTN | Std Dev |
|---------|----------------------|-----------|-----|---------|
|         | 7(S)-OH-ART (2) | 7(R)-OH-ART (3) | 6a-OH-ART (4) |          |          |
| FL#62  | 83%  | 10% | 7% | 78% (S) | 339 | 12 |
| IV-H4  | 100% | 0% | 0% | 100% (S) | 362 | 15 |
| I-A1   | 100% | 0% | 0% | 100% (S) | 49  | 3  |
| I-E2   | 100% | 0% | 0% | 100% (S) | 21  | 2  |
| V-H2   | 96%  | 0% | 4% | 100% (S) | 434 | 21 |
| III-H2 | 95%  | 0% | 5% | 100% (S) | 61  | 7  |
| IV-C11 | 93%  | 0% | 7% | 100% (S) | 454 | 17 |
| I-G12  | 92%  | 0% | 8% | 100% (S) | 413 | 6  |
| I-H4   | 92%  | 0% | 8% | 100% (S) | 498 | 23 |
| I-D12  | 92%  | 0% | 8% | 100% (S) | 287 | 8  |
| VI-C2  | 91%  | 0% | 9% | 100% (S) | 29  | 4  |
| I-B12  | 91%  | 0% | 9% | 100% (S) | 497 | 32 |
| I-C12  | 89%  | 0% | 11%| 100% (S) | 136 | 12 |
| III-A11| 89%  | 11%| 0% | 79% (S)  | 569 | 22 |
| I-B4   | 89%  | 0% | 11%| 100% (S) | 184 | 12 |
| II-B12 | 81%  | 0% | 19%| 100% (S) | 61  | 11 |
| III-D4 | 80%  | 20%| 0% | 59% (S)  | 388 | 16 |
| I-F5   | 79%  | 15%| 6% | 69% (S)  | 216 | 7  |
| I-D2   | 79%  | 0% | 21%| 100% (S) | 134 | 8  |
| III-H10| 78%  | 22%| 0% |         | 190 | 21 |
| III-A5 | 78%  | 22%| 0% |         | 421 | 27 |
| III-B2 | 77%  | 23%| 0% |         | 463 | 19 |
|                | 77%   | 23%   | 60% (S) | 416   | 25    |
|----------------|-------|-------|---------|-------|-------|
| VI-G2          | 77%   | 20%   | 0%      | 475   | 16    |
| II-C3          | 76%   | 24%   | 2%      | 602   | 16    |
| II-H11         | 74%   | 18%   | 7%      | 103   | 10    |
| VI-E10         | 72%   | 28%   | 0%      | 463   | 20    |
| IV-C1          | 70%   | 28%   | 2%      | 462   | 21    |
| II-D12         | 66%   | 22%   | 11%     | 22    | 6     |
| VI-F8          | 66%   | 31%   | 3%      | 132   | 11    |
| II-D1          | 65%   | 35%   | 0%      | 452   | 18    |
| III-H4         | 64%   | 30%   | 6%      | 134   | 13    |
| III-C10        | 63%   | 32%   | 4%      | 124   | 7     |
| I-D1           | 62%   | 38%   | 0%      | 138   | 16    |
| VI-F2          | 61%   | 33%   | 6%      | 105   | 11    |
| IV-A11         | 61%   | 32%   | 7%      | 160   | 17    |
| IV-F7          | 60%   | 34%   | 6%      | 119   | 12    |
| I-C10          | 59%   | 32%   | 8%      | 153   | 9     |
| II-H10         | 0%    | 100%  | 0%      | 270   | 8     |
| III-B1         | 19%   | 81%   | 0%      | 403   | 17    |
| I-B1           | 20%   | 75%   | 5%      | 388   | 15    |
| II-E1          | 49%   | 51%   | 0%      | 426   | 23    |
| III-B7         | 51%   | 49%   | 0%      | 75    | 4     |
| V-D8           | 53%   | 47%   | 0%      | 428   | 19    |
| IV-E10         | 53%   | 47%   | 0%      | 152   | 10    |
| IV-C3          | 58%   | 42%   | 0%      | 422   | 13    |
| II-H2          | 56%   | 40%   | 4%      | 67    | 3     |
| II-E2          | 22%   | 30%   | 48%     | 393   | 25    |
| II-A2          | 38%   | 29%   | 34%     | 21    | 6     |
| III-F1         | 46%   | 21%   | 33%     | 45    | 6     |
| II-D11         | 0%    | 0%    | 0%      | 0     | 0     |
Table S4. Catalytic turnovers and regioselectivity in artemisinin oxidation of II-E2-derived P450 variants. II-E2 is included for comparison. The group highlighted in red corresponds to the ART-reactive variants identified among the 25 top-scoring P450s based on the fingerprint-driven predictions (ordered based on 6a-selectivity), with the underlining indicating those found among the 10 top-scoring ones. The groups highlighted in green and gray correspond to the ART-reactive variants found within the bottom 50% and bottom 10% fraction, respectively, of the 50-enzyme pool. Those highlighted in darker red were selected for further characterization (Table 1 in main text: [II-E2] II-G1 = X-E12; [II-E2] III-E3 = X-F11).

| Variant       | Product distribution | TTN | StdDev |
|---------------|----------------------|-----|--------|
|               | 7(S)-OH-ART (2)      |     |        |
|               | 7(R)-OH-ART (3)      |     |        |
|               | 6a-OH-ART (4)        |     |        |
| II-E2         | 22% 30% 48%          | 393 | 25     |
| [II-E2] II-G1 (=“X-E12”) | 4% 2% 94% | 113 | 12     |
| [II-E2] III-E3 (=“X-F11”) | 0% 8% 92% | 376 | 19     |
| [II-E2] III-D2 | 0% 8% 92% | 46  | 2      |
| [II-E2] XIV-B9 | 5% 5% 90% | 87  | 6      |
| [II-E2] VI-B10 | 0% 12% 88% | 149 | 12     |
| [II-E2] VI-H4 | 3% 12% 86% | 70  | 4      |
| [II-E2] II-E12 | 6% 9% 84% | 115 | 17     |
| [II-E2] XVII-E10 | 11% 8% 81% | 171 | 9      |
| [II-E2] II-D10 | 0% 23% 77% | 105 | 11     |
| [II-E2] VI-F4 | 11% 27% 63% | 45  | 6      |
| [II-E2] XII-F10 | 0% 43% 57% | 88  | 8      |
| [II-E2] II-A5 | 16% 33% 51% | 58  | 7      |
| [II-E2] XIII-B2 | 91% 0% 9% | 474 | 23     |
| [II-E2] VII-D3 | 0% 16% 84% | 86  | 2      |
| [II-E2] XV-E12 | 6% 13% 80% | 59  | 9      |
| [II-E2] VI-A2 | 0% 21% 79% | 126 | 8      |
Materials, Methods, and Experimental Procedures

Reagents and Analytical Methods. Chemical reagents, substrates and solvents were purchased from Sigma-Aldrich, TCI, and Fluka. Silica gel chromatography purifications were carried out using AMD Silica Gel 60 230-400 mesh. UPLC/UV analyses were carried out on a Agilent 1200 spectrometer. 1D and 2D NMR experiments were carried out on a Bruker 500 MHz spectrometer. Data for $^1$H NMR spectra are reported in the conventional form: chemical shift (δ ppm), multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad), coupling constant (Hz), integration). Data for $^{13}$C NMR spectra are reported in terms of chemical shift (δ ppm). The oligonucleotides for the mutagenesis experiments were obtained from IDT DNA Technology. Restriction enzymes were purchased from New England Biolabs.

Derivatization and HPLC analysis of hydroxylated artemisinin products. To enable accurate measurement of the catalytic turnovers and regio/stereoselectivity of the P450 variants, the hydroxy-artemisinin products from the enzymatic reactions were derivatized with benzoyl chloride according to the scheme below followed by HPLC analysis of the resulting benzoyl ester derivatives.

Prior to analysis, the enzymatic reaction mixtures (0.5 mL) were added with 9-fluorenone (final conc.: 0.5 mM) as internal standard (ISTD) and extracted with 200 μL dichloromethane. After evaporation of the organic solvent in vacuo, the crude product was re-dissolved in 100 μL dry
dichloromethane and reacted with excess of benzoyl chloride (1.25 μmol; 5 equiv.) in the presence of 4-dimethylaminopyridine (2.5 μmol; 10 equiv.) for 3 hours at room temperature to give the corresponding benzoylated derivatives. After evaporation of the solvent, the residue was dissolved in 100 μL acetonitrile and the mixture was separated by high-pressure reverse-phase chromatography using an Agilent 1200 UPLC instrument equipped with a photodiode UV-VIS array. Analytical conditions: Agilent XDB-C18 column (1.8 μm, 4.4 X 50 mm); UV detection: 230 nm; flow rate: 0.8 mL/min; solvent A: 1% trifluoroacetic acid in H2O; solvent B: 1% trifluoroacetic acid in acetonitrile; gradient: 0-1 min: 20% B; 1-8 mins: 20% B to 90% B; 8-10 mins: 90% B. Retention times: 7.12 mins for 9-fluorenone (ISTD); 7.55 mins for 6a-OBz; 7.85 mins for 7(R)-OBz; 8.05 mins for 7(S)-OBz. Calibration curves were generated for each of the three hydroxylated artemisinin isomers using known concentrations of these compounds varying from 5 μM to 500 μM (Figure S6). The method showed a linear dependence of the signal (= analyte peak area/internal standard peak area) on the analyte concentration with a R-square value > 0.98 (see plots below) across the range of analyte concentration that were relevant for the enzyme characterization (2 μM – 1 mM). The detection limit of the method was determined to be about 2 μM.