Supporting Information

Single mutations toggle the substrate selectivity of multifunctional *Camptotheca* secologanic acid synthases (CYP72As)

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Tables S1-S12
Figures S1-S8
| GenBank Accession ID | Species                  | Identifiers and Notes                                         | Label Used in Phylogenetic Trees |
|---------------------|--------------------------|---------------------------------------------------------------|----------------------------------|
| MN815881            | Camptotheca acuminata    | Secologanic acid synthase; CYP72A564                          | Caa SLAS CYP72A564 | MN815881 |
| MN815882            | Camptotheca acuminata    | Secologanic acid synthase; CYP72A565                          | Caa SLAS CYP72A565 | MN815882 |
| MN815883            | Camptotheca acuminata    | Secologanic acid synthase-like; CYP72A730                      | Caa CYP72A730 | MN815883 |
| Q05047.1            | Catharanthus roseus      | Secologanin synthase; CYP72A1                                  | Cra SLS CYP72A1 | Q05047.1 |
| AGX93062.1          | Catharanthus roseus      | 7-deoxyloganic acid hydroxylase; CYP72A224                     | Cra 7DLH | AGX93062.1 |
| AGX93046.1          | Amsonia hubrichtii       | Secologanin synthase-like protein                              | Ahu SLS | AGX93046.1 |
| AGX93058.1          | Amsonia hubrichtii       | 7-deoxyloganic acid hydroxylase-like protein                   | Ahu 7DLH | AGX93058.1 |
| AGX93045.1          | Cinchona calisaya        | Secologanin synthase-like protein                              | Cca SLS | AGX93045.1 |
| AGX93057.1          | Cinchona calisaya        | 7-deoxyloganic acid hydroxylase-like protein                   | Cca 7DLH | AGX93057.1 |
| AGX93044.1          | Lonicera japonica        | Secologanin synthase-like protein                              | Lja SLS | AGX93044.1 |
| AGX93056.1          | Lonicera japonica        | 7-deoxyloganic acid hydroxylase-like protein                   | Lja 7DLH | AGX93056.1 |
| AQW38832.1          | Nothapodytes nimmoniana  | Secologanin synthase-like protein                              | Nni SLS | AQW38832.1 |
| ATN39844.1          | Olea europaea            | 7-deoxyloganic acid hydroxylase-like protein                   | Oeu 7DLH | ATN39844.1 |
| BAP90521.1          | Ophiorrhiza pumila       | Secologanin synthase                                          | Opu SLS | BAP90521.1 |
| AGX93047.1          | Rauvolfia serentina      | Secologanin synthase-like protein                              | Rsa SLS | AGX93047.1 |
| AGX93059.1          | Rauvolfia serentina      | 7-deoxyloganic acid hydroxylase-like protein                   | Rsa 7DLH | AGX93059.1 |
| BBI55002.1          | Swertia japonica         | Secologanin synthase                                          | Sja SLS | BBI55002.1 |
| AGX93048.1          | Tabernaemontana elegans  | Secologanin synthase-like protein                              | Tel SLS | AGX93048.1 |
| AGX93060.1          | Tabernaemontana elegans  | 7-deoxyloganic acid hydroxylase-like protein                   | Tel 7DLH | AGX93060.1 |
| AGX93049.1          | Vinca minor              | Secologanin synthase-like protein                              | Vmi SLS | AGX93049.1 |
| AGX93061.1          | Vinca minor              | 7-deoxyloganic acid hydroxylase-like protein                   | Vmi 7DLH | AGX93061.1 |
| NP_180239.1         | Arabidopsis thaliana     | CYP734A1; outgroup used to root the tree                       | At CYP734A1 | NP_180239.1 |
Table S2. Primers used in this study.

| Name                        | Sequence (5’→3’)                                                                 |
|-----------------------------|---------------------------------------------------------------------------------|
| Caa SLAS NdeI 5’            | ATTACATATGGGAATTCAATGGACGTCTGTCG                                              |
| CYP72A564 His6 XbaI 3’      | TAATTCTAGATCGTGGTTGGTTGGTTGGTGCGATTTGAGCCTGCG                                  |
| CYP72A564 H131F fwd         | TAACAACTTTAAAAGAATATTTCATGCATACAGTCCGTTGACCAAG                                  |
| CYP72A564 H132D fwd         | TAACAACTTTAAAAGAATGCATACAGTCCGTTGACCAAG                                       |
| CYP72A564 H131F,H132D fwd   | CAACCAACTTTAAAAGAATTtgcATGCATACAGTCCGTTGACCAAG                                  |
| CYP72A564 SRS1 rev          | GTTATGGTGACAGATCATCAATCCCTTTG                                                 |
| CYP72A564 R270T fwd         | AATCaccGAGAGGTGGTCATCGATACAGTCCG                                               |
| CYP72A564 S324E fwd         | CATGgaATGCAAGCTTTCTCTACTTCG                                                    |
| CYP72A564 S324E rev         | GATGACATCTCCATCAATCCCTTTG                                                     |
| CYP72A565 His6 XbaI 3’      | TAATTCTAGATCGTGGTTGGTTGGTTGGTGACATTTGAGCCTGCG                                  |
| CYP72A565 H131F fwd         | CAACGACTTTCAAAGAATTTTCATGCATACAGTCCG                                           |
| CYP72A565 H132D fwd         | GAATCACGCCATACAACCCACTACCAAATTC                                               |
| CYP72A565 H131F,H132D fwd   | CAACCAACGACTTTCAATGAGAATTTTCATGCATACAGTCCG                                    |
| CYP72A565 SRS1 rev          | GTTGTGGTGACAGATCATCAATCCCTTTG                                                 |
| CYP72A565 K270T fwd         | GATAaccGACAGGTGGTCGGGATCATCA                                                  |
| CYP72A565 S324E fwd         | CATGgaATGCAAGCTTTCTCTACTTCG                                                    |
| CYP72A565 S324E rev         | GATGACATCTCCATCAATCCCTTTG                                                     |
| SLS, SLAS Ancestor fwd      | ATTATCGTGGAGATGGTTTCAATCCAGTTGACATTTG                                      |
| SLS, SLAS Ancestor rev      | ATTATCGTGGAGATGGTTTCAATCCAGTTGACATTTG                                      |

Lowercase text denotes nucleotide mutation. Highlighted text denotes mutated codon.
Table S3. 95% Confidence Intervals of CYP72A564 Substrate Binding Isotherms.

| Substrate | Mutation       | $K_s / \text{mM}$ | $\Delta A_{\text{max}} / \text{mA.U.}$ | Group |
|-----------|----------------|-------------------|----------------------------------------|-------|
|           |                | Lower             | Upper                                  |       |
| loganic   | Wild Type      | 0.763             | 1.003                                  | a     |
| acid      | H131F          | --                | 0.002                                  | 1.5   |
|           | H132D          | --                | --                                     | 2.2   |
|           | H131F,H132D    | --                | 0.579                                  | 0.1   |
|           | K270T          | 0.916             | 2.561                                  | a,b   |
|           | S324E          | 1.157             | 8.284                                  | b     |
| loganin   | Wild Type      | 0.218             | 0.302                                  | a     |
|           | H131F          | 0.048             | 0.062                                  | b     |
|           | H132D          | 1.097             | 1.302                                  | b     |
|           | H131F,H132D    | 0.232             | 0.286                                  | a     |
|           | K270T          | 0.354             | 0.389                                  | d     |
|           | S324E          | 0.767             | 2.421                                  | c     |
| Substrate | Mutation     | $K_s$ / mM | $\Delta A_{\text{max}}$ / mA.U. |
|-----------|--------------|------------|-------------------------------|
|           |              | Lower      | Upper Group       | Lower      | Upper Group       |
| loganic acid | Wild Type    | 1.77       | 3.47             | ---        | 35.7               |
|            | H131F        | --         | 0.01             | 0.4        |
|            | H132D        | --         | 0.98             | --         | 4.3               |
|            | H131F,H132D  | --         | 0.02             | 0.7        | 1.4               |
|            | K270T        | 1.06       | 4.65             | 15.2       | 40.8               |
|            | S324E        | --         | --               | --         | --                |
| loganin    | Wild Type    | 0.700      | 1.016            | 46.5       | 56.2               |
|            | H131F        | 0.256      | 0.312            | 56.1       | 60.3               |
|            | H132D        | 1.596      | 10.707           | 14.2       | 60.6               |
|            | H131F,H132D  | 0.264      | 0.453            | 38.4       | 47.3               |
|            | K270T        | 1.039      | 1.395            | 31.1       | 36.8               |
|            | S324E        | 0.540      | 1.864            | 13.1       | 25.5               |
| Substrate | Mutation       | $K_M/\text{mM}$ | $V_{\text{max}}/\text{µM min}^{-1}$ |
|-----------|----------------|-----------------|-----------------------------------|
|           |                | Lower | Upper | Group | Lower | Upper | Group |
| loganic acid | Wild Type     | 1.53  | 5.20  | a      | 2.65  | 5.00  | a    |
|            | H131F         | --    | --    | --     | --    | --    | --   |
|            | H132D         | --    | --    | --     | --    | --    | --   |
|            | H131F,H132D   | --    | --    | --     | --    | --    | --   |
|            | R270T         | 2.64  | 4.91  | a      | 4.28  | 6.01  | a    |
|            | S324E         | --    | --    | --     | --    | --    | --   |
| loganin    | Wild Type     | 0.197 | 0.296 | a      | 2.84  | 3.16  | a    |
|            | H131F         | 0.041 | 0.068 | b      | 1.89  | 2.06  | b    |
|            | H132D         | 1.317 | 1.582 | c      | 3.28  | 3.55  | c    |
|            | H131F,H132D   | 0.131 | 0.922 | a,d    | 1.21  | 2.13  | b,d  |
|            | R270T         | 0.519 | 2.017 | c,d    | 3.84  | 6.55  | d    |
|            | S324E         | 1.063 | 1.485 | c      | 2.34  | 2.69  | c    |
| Substrate  | Mutation     | $K_M$ / mM |      | $V_{max}$ / μM min$^{-1}$ |      |
|------------|--------------|------------|------|--------------------------|------|
|            |              | Lower      | Upper| Group        | Lower | Upper    | Group |
| loganic acid | Wild Type    | 2.80       | 4.64 | a            | 4.27  | 5.63     | a     |
|            | H131F        | --         | --   | --           | --    | --       | --    |
|            | H132D        | --         | --   | --           | --    | --       | --    |
|            | H131F,H132D  | --         | --   | --           | --    | --       | --    |
|            | K270T        | --         | --   | --           | --    | --       | --    |
|            | S324E        | --         | --   | --           | --    | --       | --    |
| loganin    | Wild Type    | 0.578      | 0.865| a            | 3.54  | 4.09     | a     |
|            | H131F        | 0.119      | 0.457| a            | 1.61  | 2.24     | b     |
|            | H132D        | 0.966      | 9.687| b,c          | 2.58  | 7.72     | a,c,d |
|            | H131F,H132D  | 1.501      | 1.920| b            | 6.78  | 7.57     | c     |
|            | K270T        | 0.425      | 3.526| a,b,c        | 1.65  | 3.52     | b,d   |
|            | S324E        | 2.155      | 5.006| c            | 3.79  | 5.90     | a     |
Table S7. Tukey’s HSD pairwise comparison of secologanic acid production by CYP72A564 mutants.

| Comparison          | Difference in Means | SEM    | q Value  | Probability | Alpha | Significant? |
|---------------------|---------------------|--------|----------|-------------|-------|--------------|
| WT - P450           | 0.9838              | 0.09348| 14.88    | 7.7E-07     | 0.05  | Yes          |
| H131F - P450        | -0.006220           | 0.09348| 0.09412  | 1.00        | 0.05  | No           |
| H131F WT            | -0.9900             | 0.09348| 14.98    | 7.1E-07     | 0.05  | Yes          |
| H132D - P450        | 0.9516              | 0.09348| 14.40    | 1.2E-06     | 0.05  | Yes          |
| H132D WT            | -0.03213            | 0.09348| 0.4861   | 1.00        | 0.05  | No           |
| H132D H131F         | 0.9579              | 0.09348| 14.49    | 1.1E-06     | 0.05  | Yes          |
| H131F,H132D - P450  | 0.02484             | 0.09348| 0.3758   | 1.00        | 0.05  | No           |
| H131F,H132D WT      | -0.9589             | 0.09348| 14.51    | 1.1E-06     | 0.05  | Yes          |
| H131F,H132D H131F   | 0.03106             | 0.09348| 0.4699   | 1.00        | 0.05  | No           |
| H131F,H132D H132D   | -0.9268             | 0.09348| 14.02    | 1.7E-06     | 0.05  | Yes          |
| R270T - P450        | 0.7681              | 0.09348| 11.62    | 1.6E-05     | 0.05  | Yes          |
| R270T WT            | -0.2157             | 0.09348| 3.263    | 0.31        | 0.05  | No           |
| R270T H131F         | 0.7743              | 0.09348| 11.71    | 1.5E-05     | 0.05  | Yes          |
| R270T H132D         | -0.1836             | 0.09348| 2.777    | 0.48        | 0.05  | No           |
| R270T H131F,H132D   | 0.7432              | 0.09348| 11.24    | 2.4E-05     | 0.05  | Yes          |
| S324E - P450        | 0.3929              | 0.09348| 5.944    | 0.012       | 0.05  | Yes          |
| S324E WT            | -0.5908             | 0.09348| 8.938    | 3.0E-04     | 0.05  | Yes          |
| S324E H131F         | 0.3992              | 0.09348| 6.038    | 0.011       | 0.05  | Yes          |
| S324E H132D         | -0.5587             | 0.09348| 8.452    | 5.2E-04     | 0.05  | Yes          |
| S324E H131F,H132D   | 0.3681              | 0.09348| 5.569    | 0.019       | 0.05  | Yes          |
| S324E R270T         | -0.3751             | 0.09348| 5.675    | 0.017       | 0.05  | Yes          |
Table S8. Tukey’s HSD pairwise comparison of secologanic acid production by CYP72A565 mutants.

| Comparison          | Difference in Means | SEM   | q Value | Probability | Alpha | Significant? |
|---------------------|---------------------|-------|---------|-------------|-------|--------------|
| WT-P450             | 0.9886              | 0.04183 | 33.42  | < 1E-9      | 0.05  | Yes          |
| H131F -P450         | 0.004620            | 0.04183 | 0.1560 | 1           | 0.05  | No           |
| H131F WT            | -0.9839             | 0.04183 | 33.26  | < 1E-9      | 0.05  | Yes          |
| H132D -P450         | 0.5355              | 0.04183 | 18.10  | 6.4E-08     | 0.05  | Yes          |
| H132D WT            | -0.4530             | 0.04183 | 15.31  | 5.2E-07     | 0.05  | Yes          |
| H132D H131F         | 0.5309              | 0.04183 | 17.95  | 6.9E-08     | 0.05  | Yes          |
| H131F,H132D -P450   | 6.430E-04           | 0.04183 | 0.02174 | 1           | 0.05  | No           |
| H131F,H132D WT      | -0.9879             | 0.04183 | 33.40  | < 1E-9      | 0.05  | Yes          |
| H131F,H132D H131F   | -0.003970           | 0.04183 | 0.1343 | 1           | 0.05  | No           |
| H131F,H132D H132D   | -0.5349             | 0.04183 | 18.08  | 6.4E-08     | 0.05  | Yes          |
| K270T -P450         | 0.2373              | 0.04183 | 8.020  | 8.8E-04     | 0.05  | Yes          |
| K270T WT            | -0.7513             | 0.04183 | 25.40  | 1.5E-07     | 0.05  | Yes          |
| K270T H131F         | 0.2326              | 0.04183 | 7.864  | 0.0011      | 0.05  | Yes          |
| K270T H132D         | -0.2983             | 0.04183 | 10.08  | 8.2E-05     | 0.05  | Yes          |
| K270T H131F,H132D   | 0.2366              | 0.04183 | 7.998  | 9.0E-04     | 0.05  | Yes          |
| S324E -P450         | 0.3897              | 0.04183 | 13.18  | 3.7E-06     | 0.05  | Yes          |
| S324E WT            | -0.5988             | 0.04183 | 20.24  | 1.2E-07     | 0.05  | Yes          |
| S324E H131F         | 0.3851              | 0.04183 | 13.02  | 4.2E-06     | 0.05  | Yes          |
| S324E H132D         | -0.1458             | 0.04183 | 4.928  | 0.044       | 0.05  | Yes          |
| S324E H131F,H132D   | 0.3891              | 0.04183 | 13.15  | 3.7E-06     | 0.05  | Yes          |
| S324E K270T         | 0.1525              | 0.04183 | 5.155  | 0.033       | 0.05  | Yes          |
| Comparison        | Difference In Means | SEM   | q Value     | Probability | Alpha | Significant? |
|-------------------|---------------------|-------|-------------|-------------|-------|--------------|
| WT - P450         | 0.9997              | 0.09585 | 14.74984   | 8.7E-7      | 0.05  | Yes          |
| H131F - P450      | 1.8468              | 0.09585 | 27.24808   | 4.1E-8      | 0.05  | Yes          |
| H131F - WT        | 0.8471              | 0.09585 | 12.49824   | 6.9E-6      | 0.05  | Yes          |
| H132D - P450      | 0.08288             | 0.09585 | 1.22285    | 0.97        | 0.05  | No           |
| H132D - WT        | -0.91682            | 0.09585 | 13.52699   | 2.6E-6      | 0.05  | Yes          |
| H132D - H131F     | -1.76392            | 0.09585 | 26.02523   | 3.9E-6      | 0.05  | Yes          |
| H131F - H132D     | 0.79978             | 0.09585 | 11.80012   | 1.4E-5      | 0.05  | Yes          |
| H131F - WT        | -0.19992            | 0.09585 | 2.94972    | 0.41        | 0.05  | No           |
| H131F - H132D     | -1.04702            | 0.09585 | 15.44796   | 4.7E-7      | 0.05  | Yes          |
| H131F - H131F     | 0.7169              | 0.09585 | 10.57727   | 4.8E-5      | 0.05  | Yes          |
| R270T - P450      | 0.78529             | 0.09585 | 11.58633   | 1.7E-5      | 0.05  | Yes          |
| R270T - WT        | -0.21441            | 0.09585 | 3.16352    | 0.34        | 0.05  | No           |
| R270T - H131F     | -1.06151            | 0.09585 | 15.66176   | 3.8E-7      | 0.05  | Yes          |
| R270T - H132D     | 0.70241             | 0.09585 | 10.36347   | 6.0E-5      | 0.05  | Yes          |
| R270T - H131F     | -0.01449            | 0.09585 | 0.2138     | 1.00        | 0.05  | No           |
| S324E - P450      | 0.19991             | 0.09585 | 2.94955    | 0.41        | 0.05  | No           |
| S324E - WT        | -0.79979            | 0.09585 | 11.80029   | 1.4E-5      | 0.05  | Yes          |
| S324E - H131F     | -1.64689            | 0.09585 | 24.29853   | 1.7E-6      | 0.05  | Yes          |
| S324E - H132D     | 0.11703             | 0.09585 | 1.7267     | 0.87        | 0.05  | No           |
| S324E - H131F     | -0.59987            | 0.09585 | 8.85057    | 3.3E-4      | 0.05  | Yes          |
| S324E - R270T     | -0.58538            | 0.09585 | 8.63678    | 4.2E-4      | 0.05  | Yes          |
Table S10. Tukey’s HSD pairwise comparison of secologanin production by CYP72A565 mutants.

| Comparison            | Difference in Means | SEM  | q Value | Probability | Alpha | Significant? |
|-----------------------|---------------------|------|---------|-------------|-------|--------------|
| WT - P450             | 0.99927             | 0.03613 | 39.10973 | < 1E-9     | 0.05  | Yes          |
| H131F - P450          | 1.20867             | 0.03613 | 47.30507 | < 1E-9     | 0.05  | Yes          |
| H131F WT              | 0.2094              | 0.03613 | 8.19534  | 7.1E-4     | 0.05  | Yes          |
| H132D - P450          | 0.05168             | 0.03613 | 2.0226   | 0.78       | 0.05  | No           |
| H132D WT              | -0.94759            | 0.03613 | 37.08713 | < 1E-9     | 0.05  | Yes          |
| H132D H131F           | -1.15699            | 0.03613 | 45.28247 | < 1E-9     | 0.05  | Yes          |
| H131F,H132D - P450    | 0.45526             | 0.03613 | 17.81791 | 7.4E-8     | 0.05  | Yes          |
| H131F,H132D WT        | -0.54402            | 0.03613 | 21.29182 | 1.3E-7     | 0.05  | Yes          |
| H131F,H132D H131F     | -0.75341            | 0.03613 | 29.48717 | 3.9E-8     | 0.05  | Yes          |
| H131F,H132D H132D     | 0.40358             | 0.03613 | 15.79531 | 3.4E-7     | 0.05  | Yes          |
| K270T - P450          | 0.35981             | 0.03613 | 14.08213 | 1.5E-6     | 0.05  | Yes          |
| K270T WT              | -0.63947            | 0.03613 | 25.02759 | 1.4E-7     | 0.05  | Yes          |
| K270T H131F           | -0.84886            | 0.03613 | 33.22294 | < 1E-9     | 0.05  | Yes          |
| K270T H132D           | 0.30813             | 0.03613 | 12.05954 | 1.1E-5     | 0.05  | Yes          |
| K270T H131F,H132D     | -0.09545            | 0.03613 | 3.73577  | 0.19       | 0.05  | No           |
| S324E - P450          | 0.19876             | 0.03613 | 7.77905  | 1.2E-3     | 0.05  | Yes          |
| S324E WT              | -0.80051            | 0.03613 | 31.33067 | < 1E-9     | 0.05  | Yes          |
| S324E H131F           | -1.00991            | 0.03613 | 39.52602 | < 1E-9     | 0.05  | Yes          |
| S324E H132D           | 0.14708             | 0.03613 | 5.75646  | 0.015      | 0.05  | Yes          |
| S324E H131F,H132D     | -0.2565             | 0.03613 | 10.03885 | 8.6E-5     | 0.05  | Yes          |
| S324E K270T           | -0.16105            | 0.03613 | 6.30308  | 7.6E-3     | 0.05  | Yes          |
Table S11. Tukey’s HSD pairwise comparison of secologanic acid production by CYP72A564, CYP72A565, and the SLS, SLAS common ancestor.

| Comparison          | Difference in Means | SEM  | q Value    | Probability | Alpha | Significant? |
|---------------------|---------------------|------|------------|-------------|-------|--------------|
| 72A564 -P450        | 0.99635             | 0.15116 | 9.32137     | 7.75946E-4 | 0.05  | Yes          |
| 72A565 -P450        | 0.86962             | 0.15116 | 8.1358      | 0.00191     | 0.05  | Yes          |
| 72A565 72A564       | -0.12672            | 0.15116 | 1.18557     | 0.83513     | 0.05  | No           |
| Ancestor -P450      | 0.02201             | 0.15116 | 0.20591     | 0.9988      | 0.05  | No           |
| Ancestor 72A564     | -0.97434            | 0.15116 | 9.11546     | 9.02481E-4 | 0.05  | Yes          |
| Ancestor 72A565     | -0.84761            | 0.15116 | 7.92989     | 0.00226     | 0.05  | Yes          |
Table S12. Tukey’s HSD pairwise comparison of secologanin production by CYP72A564, CYP72A565, and the SLS, SLAS common ancestor.

| Comparison     | Difference in Means | SEM   | q Value   | Probability | Alpha | Significant? |
|----------------|---------------------|-------|-----------|-------------|-------|--------------|
| 72A564 -P450   | 0.99427             | 0.18904 | 7.43804  | 0.00338     | 0.05  | Yes          |
| 72A565 -P450   | 0.52359             | 0.18904 | 3.91694  | 0.09208     | 0.05  | No           |
| 72A565 72A564 | -0.47068            | 0.18904 | 3.52111  | 0.13634     | 0.05  | No           |
| Ancestor -P450| 0.04279             | 0.18904 | 0.32011  | 0.99558     | 0.05  | No           |
| Ancestor 72A564| -0.95148            | 0.18904 | 7.11794  | 0.00444     | 0.05  | Yes          |
| Ancestor 72A565| -0.4808             | 0.18904 | 3.59683  | 0.12654     | 0.05  | No           |
Figure S1. Bootstrap consensus tree containing node identifications (in parentheses) and branch lengths for the ancestral sequence reconstruction.
Figure S2. Amino acid sequence of SLS, SLAS common ancestor color coded by probability from ancestral sequence reconstruction.
Figure S3. Multiple sequence alignment of *Catharanthus* CYP72A1, *Camptotheca* CYP72A564 and CYP72A565, and their common ancestor.
Figure S4. Amino acid contacts of docked loganic acid in homology model of SLS, SLAS common ancestor.
Residues within 4.5 Å of loganic acid (green) are shown with SRSs 1-5 shown in ribbon (cyan).
Figure S5. Hydrogen bonding networks around site 324 in homology models. All residues within 4.5 Å of the amino acid at site 324 (magenta) are shown for the SLS, SLAS ancestor (A), CYP72A564 (B), CYP72A565 (C). (D) A ribbon overlay of all three models with site 324 shown. Hydrogen bonds are shown as green dotted lines.
Figure S6. Steady-state kinetics of loganic acid and loganin turnover for all CYP72A564 and CYP72A565 mutants.
Figure S7. Extracted ion chromatograms of secoiridoids from in vitro reactions.
Selective ion monitoring at m/z +397.05 [secologanic acid+Na]⁺ (A) and m/z +411.15 [secologanin+Na]⁺ (B) were used to assay secoiridoid production for CYP72A564 (left) and CYP72A565 (right) mutants. Reactions included 250 nM P450, 0.5 mU μL⁻¹ Camptotheca CPR1, 1 mM DLPC, 250 μM substrate, 500 μM NADPH, 1.00 mM glucose-6-phosphate, and 0.1 U μL⁻¹ G6PDH in 100 mM NaPO₄ (pH 7.5). Retention times: loganic acid, 10.0 min; secoiridonic acid, 11.0 min; loganin, 8.8 min; secologanin, 12.5 min.
Figure S8. Binding isotherm from SLS, SLAS common ancestor Type I binding spectra.
Binding isotherms derived by plotting the difference between the peak (389 nm) and trough (419 nm) of the difference spectra against the substrate concentration using OriginPro 2019. Table S11 records the fit parameters.