Supplemental Figure 1.

A

UV (A, UV Spectrum of FAD (red), FMN (blue)) and HPLC (B, HPLC Chromatogramm) analysis of HsaB-bound flavins after extraction from the purified protein.
A: The aerobic preparation of HsaAC exists in two oligomerization states in solution as detected by static light scattering. The octamer state of HsaAC is identified with a molecular weight of 410kD, corresponding the size of 8 to 9 copies of monomer. In addition, a second peak of molecular weight of 85kD (dimer) suggests an equilibration between dimer and octamer in solution. The gel filtration chromatography used is Superdex200 HR 10/30 column.
B: The tetrameric state of HsaA<sup>O</sup> is confirmed by static light scattering, which identified that the solution structure of HsaA<sup>O</sup> has a molecular weight of 172 kD. This corresponds to 4 copies of HsaA monomer of 43kD.
Supplemental Figure 3.

Models of the HsaA:FAD complexes. (A) Two protomers of the octameric HsaA\(^6\):FAD and (B) the tetrameric HsaA\(^0\):FAD. The positioning of FAD was based on the respective HsaA:FMN models. The C-terminal flap is highlighted in red. For clarity, a single molecule of FAD is shown.
Analytical data of metabolites.

3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione (3-HSA)

\[ R_f (n\text{-hexane/ethyl acetate} = 2:3) = 0.75. \quad \epsilon_{281\text{nm, pH 7.0}} = 2.33 \text{ mM}^{-1} \text{ cm}^{-1}. \]

GCMS \( R_f = 15.35 \) min. MS (70 eV, EI); \( m/z \): 300 (16.5%), 206 (6.5%), 193 (2.5%), 154 (11.5%), 134 (100%), 121 (17%), 107 (9.5%), 91 (7.5%), 77 (8%), 55 (8.5%).

\(^1\)H NMR (500 MHz, CDCl\(_3\)): \( \delta = 6.99 (d, \; ^3J = 8.0 \text{ Hz, 1H, 1}), 6.65 (d, \; ^3J = 2.6 \text{ Hz, 1H, 4}), 6.57 \) (dd, \(^3J = 8.0, 2.6 \text{ Hz, 1H, 2}), 4.54 (s, 1H, OH), 2.71 (“dt”, \(^3J = 12.5, 5.3 \text{ Hz, 1H, 6x}), 2.57 \) (dd, \(^2J = 19.7, \; ^3J = 9.1 \text{ Hz, 1H, 16x}), 2.53-2.41 \) (m, 4H, 6y, 8, 11xy), 2.25 (s, 3H, 19), 2.21 (dd, \(^2J = 19.4, \; ^3J = 9.1 \text{ Hz, 1H, 16y}), 2.09-1.97 \) (m, 2H, 12x, 15x), 1.93-1.82 \) (m, 2H, 14, 7x), 1.77-1.63 \) (m, 3H, 7y, 12y, 15y), 1.16 (s, 3H, 18).

\(^{13}\)C NMR (125.8 MHz, CDCl\(_3\)): \( \delta = 218.0 \) (17), 210.5 (9), 153.7 (3), 142.0 (5), 131.2 (1), 128.1 \) (10), 115.7 (4), 112.7 (2), 49.7 (8), 49.3 (14), 47.6 (13), 37.5 (11), 36.1 (16), 31.0 (6), 30.4 (12), 26.7 (7), 22.5 (15), 18.4 (19), 13.5 (18).

3-tms-HSA GCMS \( R_f = 15.06 \) min. MS (70 eV, EI); \( m/z \): 372 (20%), 206 (100%), 191 (16%), 179 (5%), 163 (3.5%), 149 (1.5%), 73 (15.5%), 55 (4%).

3,17-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9-one (3,17-DHSA)

\[ R_f (n\text{-hexane/ethyl acetate} = 2:3) = 0.5. \quad \epsilon_{280\text{nm, pH 7.0}} = 1.92 \text{ mM}^{-1} \text{ cm}^{-1}. \]

\(^1\)H NMR (500 MHz, CDCl\(_3\)): \( \delta = 6.91 (d, \; ^3J = 8.0 \text{ Hz, 1H, 1}), 6.64 (d, \; ^3J = 2.8 \text{ Hz, 1H, 4}), 6.56 \) (dd, \(^3J = 8.0, 2.8 \text{ Hz, 1H, 2}), 2.99 (“dd”, \(^3J = 19.8, 8.2 \text{ Hz, 1H}), 2.85-2.73 \) (m, 1H), 2.72-2.63 \) (m, 1H), 2.57-2.37 \) (m, 4H), 2.23 (s, 3H, 19), 1.90-1.76 \) (m, 2H), 1.73-1.63 \) (m, 1H), 1.62-1.41 \) (m, 6H), 1.20 (s, 3H, 18).

\(^{13}\)C NMR (125.8 MHz, CDCl\(_3\)): \( \delta = 211.6 \) (9), 153.6 (3), 142.1 (5), 131.1 (1), 128.1 \) (10), 115.6 \) (4), 112.6 (2), 79.6 (17), 53.5 (8), 49.5 (14), 47.0 (13), 37.9 (11), 36.1 (16), 35.3 (12), 30.9 (6), 27.9 (7), 25.6 (15), 18.4 (19), 15.1 (18).
3,17-tms-DHSA GCMS \( R_t = 15.59 \) min. MS (70 eV, EI); \( m/z \): 446 (12.5%), 206 (100%), 194 (11.5%), 191 (10%), 179 (2.5%), 163 (3.5%), 135 (2.5%), 115 (2%), 91 (1.5%), 73 (20%), 55 (2%).

3,9-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-17-one (3,9-DHSA)

\( R_f \) (n-hexane/ethyl acetate = 2:3) = 0.66.

\(^1\)H NMR (500 MHz, CDCl\(_3\)): \( \delta = 6.94 \) (d, \( J = 8.3 \) Hz, 1H, 1), 6.66 (d, \( ^3J = 2.5 \) Hz, 1H, 4), 6.57 (dd, \( J = 8.3, 2.5 \) Hz, 1H, 2), 6.4 (br s, OH(Ar)), 3.40 (“dt”, \( J = 10.4, 4.7 \) Hz, 1H, 9), 2.62-2.43 (m, 3H, 3xy, 16x), 2.19 (s, 3H,19), 2.17-2.08 (“dt”, \( J = 19.3, 9.1 \) Hz, 1H,16y), 2.01-1.89 (m, 2H, 11x, 15x), 1.82-1.69 (m, 3H, 7xy, 12x), 1.68-1.55 (m, 3H, 8, 11y, 15y), 1.44 (“dt”, \( J = 12.3, 5.7 \) Hz, 1H, 14), 1.30 (“dt”, \( J = 13.3, 4.7 \) Hz, 1H, 12y), 0.91 (s, 3H, 18).

\(^1^3\)C NMR (125.8 MHz, CDCl\(_3\)): \( \delta = 220.4 \) (17), 154.2 (3), 142.1(5), 131.0 (1), 127.3 (10), 115.6 (4), 112.7(2), 74.2 (9), 48.1 (13), 47.8 (14), 42.6 (8), 36.1 (16), 31.2 (11), 29.9 (6), 29.5 (12), 29.3 (7), 22.0 (15), 18.4 (19), 13.7 (18).

3,9-tms-DHSA GCMS \( R_t = 15.44 \) min. MS (70 eV, EI); \( m/z \): 446 (35%), 431 (4%), 356 (7.5%), 246 (6.5%), 206 (100%), 193 (26.5%), 191 (12.5%), 169 (17.5%), 163 (6%), 129 (25%), 101 (4%), 73 (45%).

3,9,17-tms-THSA (tms derivative of 3,9,17-THSA: 3,9,17-trihydroxy-9,10-seconandrost-1,3,5(10)-triene) GCMS \( R_t = 15.70 \) min. MS (70 eV, EI); \( m/z \): 520 (11%), 505 (3%), 430 (3%), 207 (22%), 206 (100%), 194 (11%), 193 (18%), 191 (8%), 169 (3%), 147 (6%), 129 (7%), 75 (8%), 73 (34%).
Analytical data of HsaAB transformation products.

3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione (3,4-DHSA) GCMS $R_t = 16.52$ min. MS (70 eV, EI); $m/z$: 316 (61.5%), 298 (2.5%), 179 (34%), 161 (4.5%), 150 (62.5%), 138 (100%), 123 (8%), 109 (7.5%), 91 (11.5%), 79 (8.5%), 55 (11.5%).

3,4-tms-DHSA GCMS $R_t = 15.91$ min. MS (70 eV, EI); $m/z$: 460 (69%), 445 (4%), 294 (50%), 281 (9%), 267 (6.5%), 251 (67.5%), 207 (10.5%), 193 (47.5%), 179 (6%), 147 (6%), 129 (8.5%), 73 (100%).

The retention time and fragmentation pattern of the HsaA-catalyzed transformation product of 3-HSA (IA) corresponded to those of 3,4-DHSA (IB) generated by incubating a culture of the ΔhsaC mutant of *R. jostii* RHA1 (1) with cholesterol (2). This showed in the Proton NMR 2 signals ($\delta = 6.57$ (d, $^3J = 8.4$ Hz, 1H, 1), $6.47$ (d, $^3J = 8.4$ Hz, 1H, 2)) corresponing to the aromatic protons.

3,4,17-tms-THSA (tms derivative of 3,4,17-THSA: 3,4,17-trihydroxy-9,10-seconandrost-1,3,5(10)-triene-9-one) GCMS $R_t = 16.37$ min. MS (70 eV, EI); $m/z$: 534 (57%), 519 (2.5%), 429 (7%), 325 (57.5%), 294 (86.5%), 282 (9.5%), 281 (9.5%), 193 (29%), 147 (5.5%), 129 (5%), 73 (100%).

3,4,9-tms-THSA (tms derivative of 3,4,9-THSA: 3,4,9-trihydroxy-9,10-seconandrost-1,3,5(10)-triene-17-one) GCMS $R_t = 16.18$ min. MS (70 eV, EI); $m/z$: 534 (65%), 444 (3%), 429 (3%), 355 (4.5%), 294 (26%), 281 (27.5%), 267 (6%), 193 (24%), 169 (10.5%), 147 (6.5%), 129 (21.5%), 73 (100%).