Electronic Supplementary Material (ESI)

Promiscuity of an Unrelated Anthrol Reductase of *Talaromyces islandicus* WF-38-12

Shailesh Kumar Singh,[†ab] Anshul Rajput,[†a] Arijit De,[a] Tapati Chakraborti [b] and Syed Masood Husain[*a]

[a]*Molecular Synthesis and Drug Discovery Unit, Centre of Biomedical Research, SGPGIMS Campus, Raebareli Road, Lucknow-226014 (India)*

[b]*Department of Biochemistry and Biophysics, University of Kalyani, Nadia-741235, West Bengal, India*

I. General Remarks

All commercial reagents were obtained from Sigma-Aldrich Chemical Co. and Sisco Research Laboratories, India. Emodin was isolated from commercially available *Rheum emodi* plant extract using reported procedure.[1] Reactions were monitored by thin-layer chromatography (TLC, 0.25 mm E. Merck silica gel plates, $60F_{254}$) and the plates were visualized by using UV light. Column chromatography was performed on silica gel 60–120/230–400 mesh obtained from S. D. Fine Chemical Co., India. Yields refer to chromatographically pure materials; conversions were calculated from the ^{1}H NMR spectra of the crude products. ^{1}H NMR spectra were recorded on Bruker 400 Ultra Shield instruments using deuterated solvents. Proton coupling constants (*J*) are reported as absolute values in Hz. ^{13}C NMR spectra were recorded on Bruker 400 Ultra Shield instruments operating at 100 MHz. Chemical shifts (δ) of the ^{1}H and ^{13}C NMR spectra are reported in ppm with a solvent resonance as an internal standard. For ^{1}H NMR: chloroform 7.26, acetone-d_6 2.05, DMSO-d_6 2.50; for ^{13}C NMR: chloroform-d_1 77.16, acetone-d_6 29.84, DMSO-d_6 39.52. The following abbreviations were used to explain the multiplicities: s = singlet, brs = broad singlet, d = doublet, dd = doublet of a doublet, ddd = doublet of a doublet of doublet, t = triplet, dt = doublet of a triplet, m = multiplet. Electrospray ionization mass spectrometry (MS) experiments were performed on an Agilent 6530 Accurate-Mass Q-TOF LC/MS system (Agilent Technologies). Optical rotations were measured on a DigiPol 781 M6U Automatic Polarimeter. CD spectroscopy was carried out on a Jasco J-1500 CD Spectrometer (Jasco International Co.) equipped with Spectra Manager™ software. UV spectroscopy and activity measurements were performed on Cary 300 UV/Vis spectrophotometer (Agilent Technologies). Optical rotation measurements were performed on Autopol III Automatic polarimeter (Rudolph Research Analytical). For determination of the enantiomeric excess (*ee*) the chiral phases Chiralcel OD-H (Daicel Inc., 250 × 4.6 mm, 5 µm), Chiralcel OJ-H (Daicel Inc., 250 × 4.6 mm, 5 µm) and Chiralpak IC (Daicel Inc., 250 × 4.6 mm, 5 µm) were used on Agilent Technologies 1260 Infinity HPLC system equipped with OpenLAB CDS v2.3 software. For determination of HPLC profile of reaction system, reverse phase columns: [Agilent Technologies Poroshell 120 EC-C18, 4 µm, 4.6 mm (ϕ) x 100 mm (L) mm)] and [Ace Excel 5 C18-AR, 5 µm, 4.6 mm (ϕ) x 250 mm (L) mm] were used on Agilent Technologies 1260 Infinity II HPLC system equipped with OpenLAB CDS v2.3 software.

II. Bioinformatic analysis of ARti-2

An NCBI non-redundant protein database BLAST search of the CRG89873.1 (represented as "ARti-2") revealed that it has a strong amino acid identity with known anthrols reductases such as 17β-hydroxysteroid dehydrogenase (17β-HSDcl) of *Curvularia lunata* (3e-171 e-value, 84% identity), Anthrol reductase (ARti) of *Talaromyces islandicus* (4e-155 e-value, 77% identity), monodictyphenone synthesis protein C (MdpC) of *Aspergillus nidulans* (2e-145 e-value, 71% identity), and AflM of *Aspergillus parasiticus* (5e-124 e-value, 67% identity); and naphthol reductases such as trihydroxy naphthalene reductase (T_3HNR) (2e-107 e-value, 59% identity) and tetrahydroxy naphthalene reductase (T_4HNR) (5e-81 e-value, 49% identity) of *Magnaporthe grisea*. CRG89873.1 was predicted to be one of the core biosynthetic genes and the part of the putative cluster of unknown type as predicted by fungiSMASH (fungal version of antiSMASH).[2] The gene cluster contained a total of 10 genes including ARti-2 and were annotated using conserved domain search (CDD v3.17) at NCBI (Table S1). However, the direct role of genes other than ARti-2 in biosynthesis and/or modification of anthraquinones could be established based on *in silico* analysis. However, CRG89872.1 (denoted as putative anthraquinone reductase) shows 57% sequence identity with AgnL4 (proposed to catalyse the reduction step of emodin to emodin hydroquinone) of agnestin biosynthesis gene cluster.[3] This hints at the possibility of CRG89872.1 to catalyze the reduction of anthraquinones to anthrols.

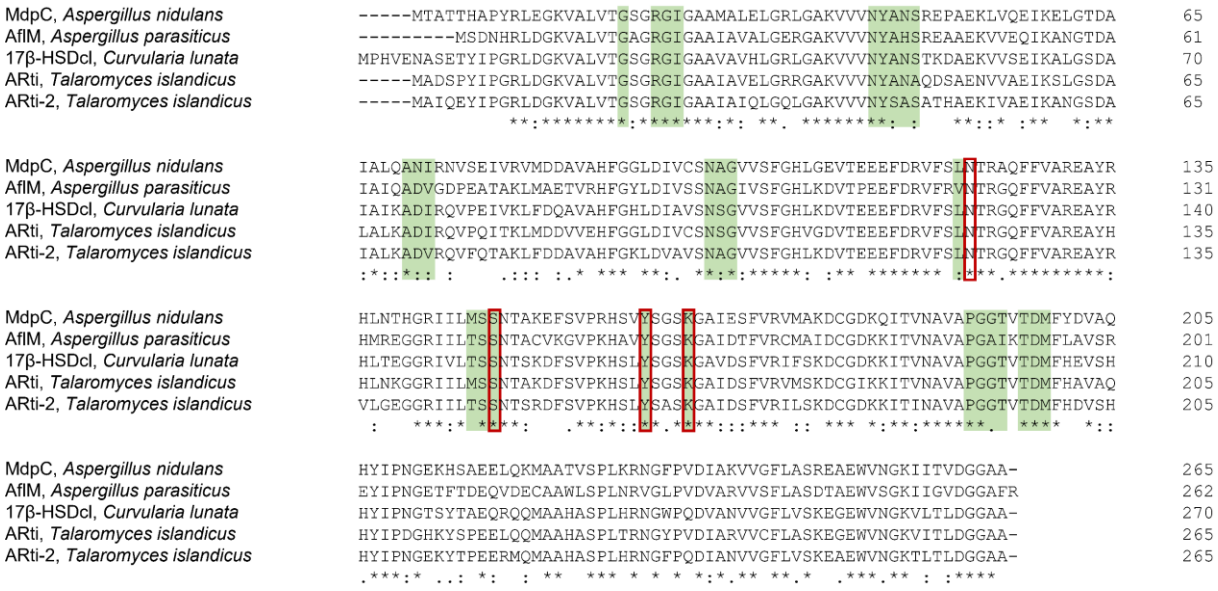


Figure S1. Multiple sequence alignment (CLUSTAL Omega 1.2.4)[4] of new SDR from *T. islandicus* (ARti-2) (*CRG89873.1*) with known anthrol reductases such as MdpC from *A. nidulans* (*Q5BH34.1*); AflM from *A. parasiticus* (*AAB42156.1*); 17β-HSD from *C. lunata* (*AAD12052.1*) and ARti from *T. islandicus* (*CRG86682.1*) reveals that they share common NAD(P)-binding site (green-shaded), identical catalytic residues (red-boxed) at the active site; suggesting similar functions. Three-dimensional information for 17β-HSDcl (PDB ID: *4FJ0*)[5] were accessed using iCn3D Structure Viewer[6] at NCBI.

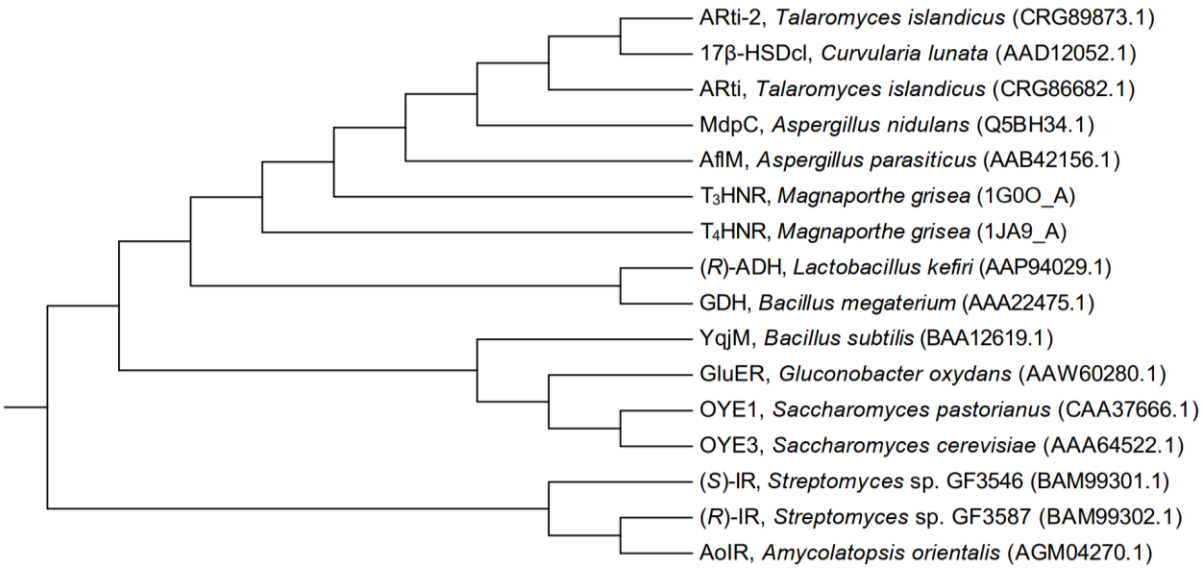
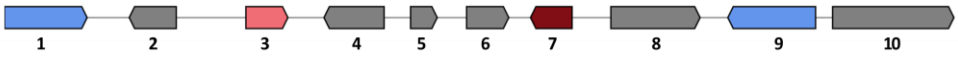


Figure S2. Phylogenetic tree of NAD(P)-dependent oxidoreductases. ARti-2 lies closest to 17β-HSDcl indicating similar function of both enzymes.

Table S1. Proposed gene functions of ARti-2 gene cluster members.



Entry	Gene (Protein Accession ID)	Conserved domain	E-value
1	CRG89867.1	Yeast Polyamine transporter 1 (Tpo1) and similar multidrug resistance (MDR) transporters	2.99e-92
2	CRG89868.1	-	-
3	CRG89869.1	classical SDR	2.02e-11
4	CRG89870.1	Fungal Zn(2)-Cys(6) binuclear cluster domain	9.22e-09
5	CRG89871.1	-	-
6	CRG89872.1 (putative anthraquinone reductase)	Flavin reductase, atypical SDR	6.13e-14
7	CRG89873.1 (**ARti-2**)	tetrahydroxynaphthalene/trihydroxynaphthalene reductase-like classical SDR	1.97e-102
8	CRG89874.1	FAD-dependent monooxygenase	5.57e-37
9	CRG89875.1	Azole resistance protein 1 (Azr1p), and similar multidrug resistance (MDR) transporters	7.92e-106
10	CRG89876.1	fungal transcription factor regulatory middle homology region	5.39e-41

Codon-optimized nucleotide sequence of ARti-2:

ATGGCTATCCAGGAATATATACCGGGTCGCCTGGATGGTAAAGTGGCCTTAGTGACGGGCTCAGGTCGCGGCA
TCGGCGCCGCTATTGCGATCCAGCTGGGTCAGTTAGGTGCCAAAGTGGTTGTTAATTATTCTGCCAGTGCTACC
CATGCCGAAAAAATCGTTGCGGAAATCAAAGCCAATGGTTCAGATGCGATCGCCCTGAAAGCAGATGTTCGTC
AGGTGTTTCAGACAGCCAAACTGTTTGATGATGCCGTTGCACATTTTGGTAAACTGGATGTGGCAGTGAGTAA
TGCAGGCGTTGTGAGCTTTGGTCATCTGAAAGATGTTACCGAAGAAGAATTTGATCGCGTGTTTAGTCTGAAT
ACACGCGGCCAGTTTTTTGTGGCACGCGAAGCCTATCGTGTGCTGGGCGAAGGCGGTCGTATTATCTTAACGA
GTAGTAATACCTCTCGTGATTTTAGCGTTCCTAAACATAGTCTGTATAGCGCAAGTAAAGGCGCCATTGATAG
CTTTGTGCGTATCCTGTCTAAAGATTGCGGCGATAAAAAAATTACGATCAATGCCGTTGCTCCGGGCGGTACG
GTTACGGATATGTTTCATGATGTGTCTCATCATTATATTCCGAATGGTGAAAAATATACACCGGAAGAACGTA

TGCAGATGGCCGCACATGCCTCTCCACTGCATCGCAATGGCTTTCCACAGGATATTGCTAATGTTGTGGGCTTT
CTGGTGAGCAAAGAAGCTGAATGGGTTAATGGCAAAACCTTAACCTTAGATGGCGGTGCTGCTCTCGAGTAA

Protein sequence of ARti-2_his:

MGHHHHHHHHHHSSGHIDDDDKHMMAIQEYIPGRLDGKVALVTGSGRGIGAAIAIQLGQLGAKVVVNYSASATH
AEKIVAEIKANGSDAIALKADVRQVFQTAKLFDDAVAHFGKLDVAVSNAGVVSFGHLKDVTEEEFDRVFSLNTRG
QFFVAREAYRVLGEGGRIILTSSNTSRDFSVPKHSLYSASKGAIDSFVRILSKDCGDKKITINAVAPGGTVTDMFHDV
SHHYIPNGEKYTPEERMQMAAHASPLHRNGFPQDIANVVGFLVSKEAEWVNGKTLTLDGGAA

III. Cloning, Expression and Purification of Enzymes

Gene synthesis and expression vector

The synthesis of codon-optimized gene encoding ARti-2 (NCBI GenBank accession: CRG89873.1) was ordered from Biomatik Company (Biomatik, Ontario, Canada). The gene was cloned into pET-19b vector using 5'-NdeI and 3'-XhoI yielding (N)*his*$_{10}$-tagged ARti-2. Plasmid encoding Glucose dehydrogenase (GDH) was obtained from Prof. Werner Hummel (University of Bielefeld, Germany).

Transformation of plasmid to E. coli cells

Transformation of plasmid DNA to competent *E. coli* BL21 (DE3) cells was performed by applying a heat shock at 42 °C for 50 s. The transformed cells were grown overnight on SOB-agar medium containing 100 µg/mL ampicillin.

Media and growth conditions

One clone was picked and dispersed in 5 mL of LB-media (Lennox), followed by incubation overnight (37 °C, 220 rpm). Ampicillin (100 µg·mL^{-1}) was added as required.

Cultivation and expression

ARti-2: The overnight cultures were diluted to 500 mL of medium each (ampicillin 100 µg·mL^{-1}) and incubated at 37 °C, 160 rpm. IPTG (0.2 mM) was added to the mid-log phase (OD_{600} = 0.6) was reached, and cultures were incubated for 20 h at 18 °C, 160 rpm.

GDH: The overnight cultures were diluted to 500 mL of medium each (ampicillin 100 µg·mL^{-1}) and incubated at 37 °C, 160 rpm. IPTG (0.2 mM) was added to the mid-log phase (OD_{600} = 0.6) was reached, and cultures were incubated for 4 h at 37 °C, 160 rpm.

Workup and storage

ARti-2: The harvested *E. coli* cells were resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0; 2.5 mL per harvested cells of 500 mL medium).

GDH: The harvested *E. coli* cells were resuspended in lysis buffer (50 mM HEPES, pH = 8.0; 2.5 mL per harvested cells of 500 mL medium).

The cells were disrupted by sonication (8 × 10 sec, Vibra-Cell Processors, model number VCX500, Sonics), followed by centrifugation (30 min, 12000×*g*, 4 °C). Glycerol (20% v/v) was added, and the crude enzyme preparation was frozen at –20 °C.

Enzyme purification

ARti-2_*his* was purified by Ni-NTA affinity chromatography. Non-specifically bound proteins were washed off with 5 mM and 20 mM imidazole in Tris buffer (25 mM Tris-HCl, pH 8.0). Elution was performed with 25 mM Tris buffer (pH = 8.0) containing 50 and 250 mM imidazole. The purity of the enzyme was confirmed by SDS-PAGE (Figure S3). The fractions containing purified proteins are collected and desalted by gel filtration (Econo-Pac 10DG desalting gel column, Bio-Rad). The concentration of the protein was performed by ultrafiltration (Vivaspin 20R centrifugal filter units, 10 kDa nominal molecular weight limit, Sartorius). The concentration of the protein was determined by measuring the UV absorption at 280 nm (NanoVue, GE Healthcare; extinction coefficient 14440 M^{-1}·cm^{-1}, molecular weight 31186 Da).

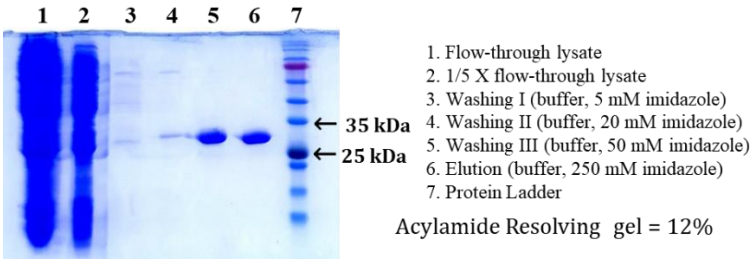


Figure S3. SDS-PAGE image of ARti-2_*his* purification by Ni-NTA affinity chromatography.

IV. Melting Temperature (T_m) and Optimum pH of ARti-2

Melting temperature (T_m) of ARti-2_his:

Ellipticity of ARti-2_*his* (0.2 mg/mL in 25 mM Tris-HCl, pH 8.0) was recorded at 222 nm as a function of temperature (25–85 °C). The data obtained were fitted to the Boltzmann equation for the determination of the melting temperature of ARti-2_*his* where the midpoint value x0 corresponds to the T_m (= 43 °C). The denatured enzyme was cooled down back to 20 °C to follow whether ARti-2 could be renatured reversibly. However, renaturation was not observed as evident from the cooling curve (Figure 2a, Main Text).

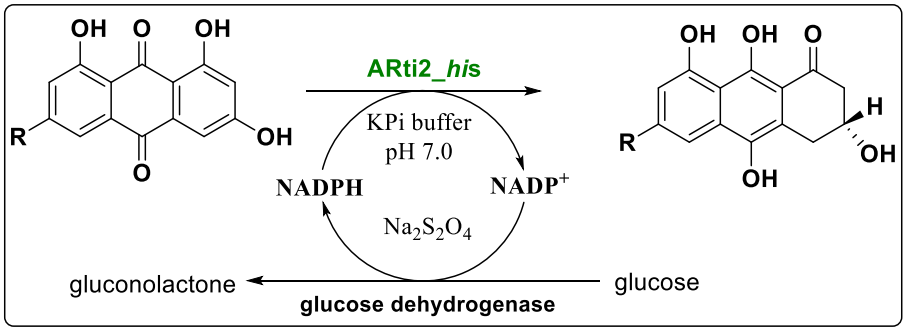
Determination of optimum pH:

For screening of optimum pH for ARti-2_*his*-catalyzed reduction of emodin hydroquinone (**10a**/**10b**), potassium phosphate buffer (3 mL, 50 mM KPi, 1 mM EDTA, 1 mM DTT) with different pH (5.5–8.0) was prepared. The buffer was degassed under reduced pressure for 20 min using argon medium. Under argon counterflow, D-glucose (55 µmol, 5 equiv.), $NADP^+$ (1.1 µmol, 0.1 equiv.), $Na_2S_2O_4$ (220 µmol, 20 equiv.), and emodin (**9**; 3 mg, 11 µmol) in DMSO (0.3 mL, 10% v/v), GDH (20 U), and ARti-2_*his* (150 µL, 1.68 mg/mL) were added to the buffer and the mixture was stirred under argon atmosphere at room temperature for 5 h. The solution was extracted with EtOAc and the solvent was removed under reduced pressure. The crude reaction mixture was analyzed by ^{1}H NMR in acetone-d_6.

Conversion: 38% (pH 5.5), 72% (pH 6.0), 93% (pH 6.5), 90% (pH 7.0), 85% (pH 7.5), 46% (pH 8.0).

V. Substrate Scope

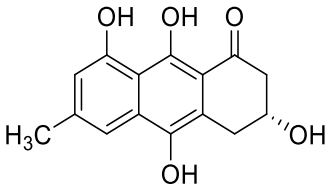
Substrates were synthesized using reported methods: tetrahydroxynaphthalene (T_4HN, **4**),[7] lunatin (**12**)[8] and citreorosein (**15**)[9].



Scheme S1. Stereo- and regioselective reduction of anthraquinones

General Reaction Procedure: To a 50 mL round bottom flask, $NADP^+$ (0.0073 mmol, 0.1 equivalent), glucose (0.365 mmol, 5 equivalent) and GDH (50 U) were added into degassed buffer (20 mL, 50 mM KPi, 1 mM DTT, 1 mM EDTA, pH 7.0) at room temperature. After that anthraquinone (0.073 mmol in DMSO, 10% v/v) was added followed by $Na_2S_2O_4$ (1.46 mmol, 20 equivalent). ARti-2_*his* (500 µL, 1.68 mg/mL) was added to the reaction mixture and kept stirring (100 rpm) for 12 h at room temperature. The reaction mixtures were subjected to extraction in EtOAc (3 x 10 mL) by vortexing and centrifugation to separate the organic and aqueous layers. The combined organic layer was dried over anhydrous Na_2SO_4, evaporated to dryness at the rotary evaporator and ^1H NMR at 400 MHz in acetone–d_6 was recorded. After that, the crude product was purified using column chromatography to afford pure dihydroanthracenone. The absolute configuration and enantiopurity of dihydroanthracenones were confirmed by HPLC. The HPLC profile of ARti-2 reaction system with various substrates was also obtained.

(*R*)-3,8,9,10-tetrahydroxy-6-methyl-3,4-dihydroanthracen-1(*2H*)-one (8)



$C_{15}H_{14}O_5$: 274.27 g/mol

Yield: 74%

TLC: (EtOAc: Hexane, 1:1 v/v): *Rf* = 0.24;

^1H NMR (400 MHz, acetone-*d*6): δ [ppm] 2.44 (s, 3H), 2.98 (ddd, *J* = 17.1 Hz, *J* = 7.1Hz, *J* = 1.1 Hz, 1H), 3.00 (dd, *J* = 17.1 Hz, *J* = 2.9 Hz, 1H), 3.06 (dd, *J* = 16.4 Hz, *J* = 6.8 Hz, 1H), 3.28 (dd, *J* = 16.4 Hz, *J* = 3.6 Hz, 1H), 4.42–4.46 (m, 2H), 6.69 (s, 1H), 7.47 (s, 1H), 7.64 (s, 1H), 9.78 (s, 1H), 15.94 (s, 1H).

^{13}C NMR (100 MHz, acetone-d_6): δ [ppm] 22.3, 32.6, 46.7, 66.0, 110.0, 111.6, 113.2, 113.4, 117.7, 134.0, 141.6, 143.7, 159.0, 160.3, 204.8.

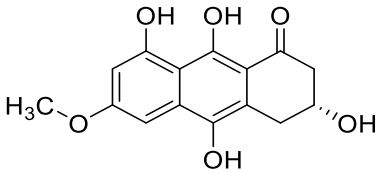
HPLC (chiral) [Flow rate: 1 mL/min; Typical injection volume: 5 µL; Isocratic: 95% n-Hexane, 5% Isopropanol; DAD: 280 nm (bandwidth = 4 nm); Column: Chiralcel OD-H, 5 µm, 4.6 mm (ϕ) x 250 mm (L) mm, Temperature: 20 °C.]: Retention time (R_t), (**8**) = 62.55 min.; >99% *ee* (determined by comparison to *rac*-**8**, R_t [(*S*)-**8**] = 52.27 min, R_t [(*R*)-**8**] = 63.23 min.

HPLC (reaction profile) [Flow rate: 0.5 mL/min; Typical injection volume: 10 µL; Isocratic: 70% acetonitrile, 30% water (with 0.2% v/v triflouroacetic acid); DAD: 430 nm (bandwidth = 4 nm); Column: Agilent Technologies Poroshell 120 EC-C18, 4 µm, 4.6 mm (ϕ) x 100 mm (L) mm, Temperature: 25 °C.]: Retention time (R_t), (**9**) = 4.26 min, (**8**) = 2.32 min.

$[\alpha]_D^{27}$ = +12.12 (acetonitrile), 0.066 g/100 mL

Exact Mass $[M+H]^+$: 275.0914 (calculated), 275.0907 (found)

(*R*)-3,8,9,10-tetrahydroxy-6-methoxy-3,4-dihydroanthracen-1(*2H*)-one (14)



$C_{15}H_{14}O_6$: 290.27 g/mol

Yield: 64 %

TLC: (EtOAc: Hexane, 1:1 v/v): *Rf* = 0.30.

^{1}H NMR (400 MHz, acetone-d_6): δ [ppm] 2.77 (dd, *J* = 17.1 Hz, *J* = 7.2 Hz, 1H), 2.98 (dd, *J* = 17.2 Hz, *J* = 3.6 Hz, 1H), 3.04 (dd, *J* = 16.4 Hz, *J* = 6.8 Hz, 1H), 3.26 (dd, *J* = 16.5 Hz, *J* = 3.4 Hz, 1H), 3.91 (s, 3H), 4.40–4.43 (m, 2H), 6.43 (d, *J* = 1.79 Hz, 1H), 7.10 (d, *J* = 1.76 Hz, 1H), 7.66 (s, 1H), 9.98 (s, 1H), 16.17 (s, 1H).

^{13}C NMR (100 MHz, Acetone-d_6): δ [ppm] 32.6, 46.4, 55.8, 66.0, 95.3, 101.7, 108.7, 109.1, 118.4, 135.5, 141.1, 160.8, 161.1, 164.2, 203.9.

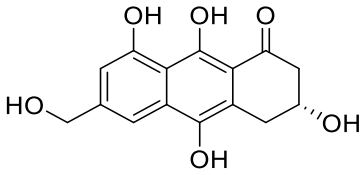
HPLC (chiral) [Flow rate: 0.8 mL/min; Typical injection volume: 5 µL; Isocratic: 92% n-Hexane, 8% Isopropanol; DAD: 280 nm (bandwidth = 4 nm); Column: Chiralcel OD-H, 5 µm, 4.6 mm (ϕ) x 250 mm (L) mm, Temperature: 20 °C.]: Retention time (R_t), (**14**) = 48.15 min.; >99% *ee* (determined by comparison to *rac*-**14**, R_t [(*S*)-**14**] = 39.21 min, R_t [(*R*)-**14**] = 48.78 min.

HPLC (reaction profile) [Flow rate: 0.5 mL/min; Typical injection volume: 10 µL; Isocratic: 70% acetonitrile, 30% water (with 0.2% v/v triflouroacetic acid); DAD: 430 nm (bandwidth = 4 nm); Column: Agilent Technologies Poroshell 120 EC-C18, 4 µm, 4.6 mm (ϕ) x 100 mm (L) mm, Temperature: 25 °C.]: Retention time (R_t), (**12**) = 3.68 min, (**14**) = 2.24 min.

$[\alpha]_D^{27}$ = +37.88 (acetonitrile), 0.066 g/100 mL

Exact Mass $[M+H]^+$: 291.0863 (calculated), 291.0877 (found).

(***R*)-3,8,9,10-tetrahydroxy-6-(hydroxymethyl)-3,4-dihydroanthracen-1(2*H*)-one (17)**



$C_{15}H_{14}O_6$: 290.27 g/mol

Yield: 72%

TLC (MeOH: $CHCl_3$, 1:9 v/v): R_f = 0.2.

^{1}H NMR (400 MHz, acetone-d_6): δ (ppm) 2.81 (dd, *J* = 17.1, 7.0 Hz, 1H), 3.01 (dd, *J* = 17.0, 3.2 Hz, 1H), 3.09 (dd, *J* = 16.5, 6.5 Hz, 1H), 3.27 (dd, *J* = 16.3, 3.3 Hz, 1H), 4.45 (m, 2H), 4.74 (s, 2H), 6.84 (s, 1H), 7.68 (s, 1H), 7.77 (s, 1H), 9.81 (s, 1H), 15.90 (s, 1H).

^{13}C NMR (100 MHz, acetone-d_6): δ (ppm) 32.6, 46.8, 64.7, 66.0, 110.0, 110.3, 110.6, 112.4, 117.8, 133.9, 142.1, 148.1, 159.0, 160.0, 205.1.

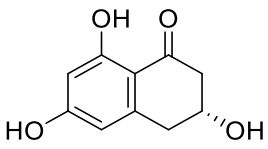
HPLC (chiral) [Flow rate: 0.5 mL/min; Typical injection volume: 5 µL; Isocratic: 85% n-Hexane, 15% Isopropanol; DAD: 280 nm (bandwidth = 4 nm); Column: Chiralpak IC, 5 µm, 4.6 mm (ϕ) x 250 mm (L) mm, Temperature: 20 °C.]: Retention time (R_t), (**17**) = 48.04 min.; >99% *ee* (determined by comparison to *rac*-**17**, R_t [(*S*)-**17**] = 41.23 min, R_t [(*R*)-**17**] = 48.66 min).

HPLC (reaction profile) [Flow rate: 0.5 mL/min; Typical injection volume: 10 µL; Isocratic: 70% acetonitrile, 30% water (with 0.2% v/v triflouroacetic acid); DAD: 430 nm (bandwidth = 4 nm); Column: Agilent Technologies Poroshell 120 EC-C18, 4 µm, 4.6 mm (ϕ) x 100 mm (L) mm, Temperature: 25 °C.]: Retention time (R_t), (**15**) = 1.90 min, (**17**) = 2.29 min.

$[\alpha]_D^{27}$ = +62.50 (acetonitrile), 0.040 g/100 mL

Exact Mass $[M+H]^+$: 291.0863 (calculated), 291.0854 (found).

(*R*)-Scytalone (6)



$C_{10}H_{10}O_4$: 194.18 g/mol

The ARti-2_*his*-catalyzed reduction was performed as follows: Argon was bubbled through the potassium phosphate buffer (50 mM KPi, 1 mM EDTA, 1 mM DTT, pH 7.0) solution for 20 min, followed by degassing

under reduced pressure. 1,3,6,8-tetrahydroxynaphthalene (**4**) (104 µmol, 20 mg) was added to a solution of D-glucose (520 µmol, 94 mg) and $NADP^+$-Na (10 µmol, 8 mg) in 10.0 mL of buffer. ARti-2_*his* (500 µL, 1.68 mg/mL) and 100 U of GDH were added slowly and the reaction was stirred under nitrogen for 12 h. The solution was extracted 3 times with EtOAc, dried over Na_2SO_4 and the solvent was removed under reduced pressure. Purification through flash column chromatography (EtOAc: Hexane = 1:1) yielded **6**.

TLC (EtOAc: Hexane, 1:1 v/v): R_f = 0.18;

Yield: 23%

^{1}H NMR: (400 MHz, acetone-d_6) δ (ppm) 2.63 (dd, *J* =17.2 Hz, *J* = 7.5 Hz, 2H), 2.81–2.91 (m, 2H), 3.11 (dd, 16.1 Hz, *J* = 3.2 Hz, 1H), 4.28–4.36 (m, 2H), 6.16 (d, *J* = 1.98 Hz, 1H), 6.29 (d, *J* = 1.96 Hz, 1H), 9.41 (s, 1H), 12.79 (s, 1H).

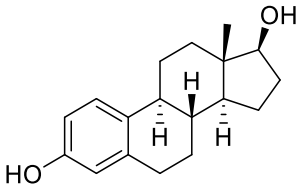
^{13}C NMR: (100 MHz, acetone-d_6), δ (ppm) 39.2, 47.5, 66.4, 101.4, 108.9, 111.7, 146.1, 165.5, 166.3, 202.5.

HPLC (chiral) [Flow rate: 0.8 mL/min; Typical injection volume: 2 µL; Isocratic: 80% n-Hexane, 20% Isopropanol; DAD: 280 nm (bandwidth = 4 nm); Column: Chiralcel OJ-H, 5 µm, 4.6 mm (ϕ) x 250 mm (L) mm, Temperature: 18 °C.]: Retention time (R_t), (**6**) = 9.75 min.; >99% *ee* (determined by comparison to *rac*-**6**, R_t [(*S*)-**6**)] = 8.94 min, R_t (*R*)-**6**] = 9.78 min).

HPLC (reaction profile) [Flow rate: 0.5 mL/min; Typical injection volume: 10 µL; Isocratic: 25% acetonitrile, 75% water (with 0.2% v/v triflouroacetic acid); DAD: 280 nm (bandwidth = 4 nm); Column: Agilent Technologies Poroshell 120 EC-C18, 4 µm, 4.6 mm (ϕ) x 100 mm (L) mm, Temperature: 25 °C.]: Retention time (R_t), (**4**) = 3.70 min, (**6**) = 3.29 min.

Exact Mass $[M+H]^+$: 195.0652 (calculated), 195.0662 (found).

17*β*-Estradiol (19)



$C_{18}H_{24}O_2$: 272.18 g/mol

TLC (MeOH: $CHCl_3$, 1:9 v/v): *Rf* = 0.43.

Yield: 15%

To a 50 mL round bottom flask, $NADP^+$ (0.0073 mmol, 0.1 equivalent), glucose (0.367 mmol, 5 equivalent) and GDH (50 U) was added into degassed buffer (20 mL, 50 mM KPi, 1 mM EDTA, 1 mM DTT, pH 7.0) at room temperature. After that estrone **18** (0.073 mmol in DMF, 1% v/v) was added. ARti-2_*his* (500 µL, 1.68 mg/mL) was added to the reaction mixture and kept stirring (100 rpm) for 12 h at room temperature. Then EtOAc (3 x 10 mL) the reaction mixtures were subjected to vortexing and centrifugation to separate the organic and aqueous

layers. The combined organic layer was dried over anhydrous Na_2SO_4, evaporate to dryness at rotary evaporator and ^1H NMR at 400 MHz in $CDCl_3$ was recorded. After that the crude product was purified using column chromatography using MeOH: DCM as eluent to afford **19** as white solid. The absolute configuration of **19** was determined by comparison with previously reported 1H NMR data.[10]

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.77 (s, 3H), 1.52–1.68 (m, 9H), 1.84–1.88 (m, 1H), 1.93–1.96 (m, 1H), 2.08–2.22 (m, 2H), 2.30 (dd, *J* = 13.2, 3.7 Hz, 1H), 2.80–2.85 (m, 1H), 3.73 (t, *J* = 8.5 Hz, 1H), 4.6 (s, 1H), 7.56 (d, *J* = 2.1 Hz, 1H), 6.62 (dd, *J* = 8.6, 2.7 Hz, 1H), 7.15 (d, *J* = 8.5 Hz, 1H).

^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 11.2, 23.3, 26.5, 27.3, 29.8, 30.7, 36.8, 39.0, 43.4, 44.1, 50.2, 82.1, 112.8, 115.4, 126.7, 132.9, 138.4, 155.3.

HPLC (reaction profile) [Flow rate: 1 mL/min; Typical injection volume: 10 µL; Isocratic: 70% acetonitrile, 30% water; DAD: 280 nm (bandwidth = 4 nm); Column: Ace Excel 5 C18-AR, 5 µm, 4.6 mm (ϕ) x 250 mm (L) mm, Temperature: 25 °C.]: Retention time (R_t), (**18**) = 4.46 min, (**19**) = 3.73 min.

Exact Mass $[M+H]^+$: 273.1849 (calculated), 273.1833 (found).

General procedure for synthesis of various racemic dihydroanthracenones (*rac*-8, *rac*-14, *rac*-17):[11]

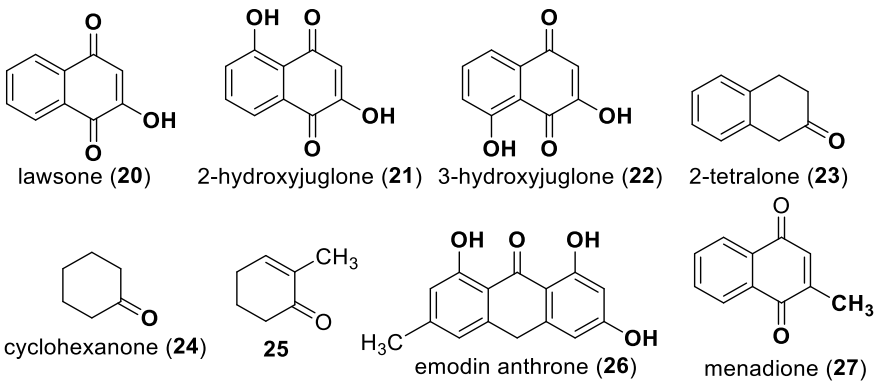
To a 50 ml round bottom flask in 10 mL degassed water the anthraquinone (**9**,**12**,**15**) (74 µmol) in methanol (10% v/v) was added in argon atmosphere. $Na_2S_2O_4$ (20 equiv., 1.48 mmol) and $NaBH_4$ (20 equiv., 1.48 mmol) were added portion-wise at ice-cold temperature. After 20 min, 1 N HCl was added and the reaction mixture was extracted in EtOAc and concentrated on rotary evaporator. The crude reaction mixture was subjected for the column chromatography and the product was purified using column chromatography.

Synthesis of racemic scytalone (*rac*-6):[7]

To a 50 ml round bottom flask in 15 mL degassed water the T_4HN (**4**) (160 µmol) in isopropanol (10% v/v) was added in argon atmosphere. $NaBH_4$ (20 equiv., 3.2 mmol) was added portion-wise at room temperature. After 20 min, 1 N HCl was added and the reaction mixture was extracted in EtOAc and concentrated on rotary evaporator. The crude reaction mixture was subjected to the column chromatography and the product racemic scytalone (*rac*-**6**) was purified using Hexane: EtOAc (3:2).

Substrates which are not reduced by ARti-2

Lawsone (**20**), 2-tetralone (**23**), cyclohexanone (**24**), 2-methyl-2-cyclohexen-1-one (**25**) and menadione (**27**) were commercially obtained. Substrates were synthesized using reported methods: 2-Hydroxyjuglone (**21**) & 3-hydroxyjuglone (**22**)[12] and emodin anthrone (**26**)[13].



General Procedure for the reduction by ARti-2

Procedure for ARti-2-catalyzed reduction of lawsone (**20**): Argon was bubbled through the potassium phosphate buffer (50 mM KPi, 1 mM EDTA, 1 mM DTT, pH 7.0) solution for 20 min, followed by degassing under reduced pressure. Lawsone (**20**) (0.114 mmol in isopropanol 10% v/v) was added to a solution of D-glucose (0.570 mmol) and $NADP^+$ (0.114 mmol) in 20.0 mL of buffer. ARti-2_*his* (500 µL, 1.68 mg/mL) and 50 U of GDH were added slowly and the reaction was stirred under nitrogen for 12 h. The solution was extracted 3 times with EtOAc, dried over Na_2SO_4 and the solvent was removed under reduced pressure. TLC and NMR show no product formation. Likewise, **21**–**25**,**27** were screened that too show no product formation.

Procedure for ARti-2-catalyzed reduction of emodin anthrone (**26**): To a 25 mL round bottom flask, $NADP^+$ (0.0037 mmol, 0.1 equivalent), glucose (0.185 mmol, 5 equivalent) and GDH (30 U) were added into degassed buffer (50 mM KPi, 1 mM EDTA, 1 mM DTT, pH 7.0) at room temperature. After that **26** (0.037 mmol in DMSO, 10% v/v) was added. ARti-2_*his* (250 µL, 1.68 mg/mL) was added to the reaction mixture and kept stirring (100 rpm) for 12 h at room temperature. The reaction mixtures were subjected to extraction in EtOAc (3 x 10 mL) by vortexing and centrifugation to separate the organic and aqueous layers. The combined organic layer was dried over anhydrous Na_2SO_4, evaporated to dryness at the rotary evaporator and ^1H NMR at 400 MHz in acetone–d_6 was recorded. NMR shows no product formation.

Substrates **21**–**25**,**27** were also tested using photometric assay as follows: 10 µL of a 20 mM NADPH solution in potassium phosphate buffer (50 mM KPi, 1 mM EDTA, 1 mM DTT, pH 7.0) & 10 µL of a 20 mM solution of substrates in 2-propanol were added to 970 µL of the same buffer and mixed. After addition of 10 µL of the enzyme preparation, the solution was mixed gently and the decrease in absorbance was measured for 1 minute at 340 nm and 25 °C. Substrate concentration and amount of ARti-2 were varied to verify the status of NADPH consumption. However, no NADPH consumption was observed.

VI. Kinetic Profile of ARti-2

A common reverse phase HPLC method was employed to determine the reaction velocity of enzyme-catalyzed reactions based on internal standard analysis:

HPLC method: [Flow rate: 0.5 mL/min; Typical injection volume: 10 µL; Isocratic: 70% acetonitrile, 30% water (with 0.2% v/v triflouroacetic acid); DAD: 430 nm (bandwidth = 4 nm); Column: Agilent Technologies Poroshell 120 EC-C18, 4 µm, 4.6 mm (ϕ) x 100 mm (L) mm, Temperature: 25 °C.]

General procedure: To a capped 2-mL microcentrifuge vial, $NADP^+$ (0.74 µmol), glucose (37 µmol) and GDH (8 U) were added into degassed buffer (1 mL, 50 mM KPi, 1 mM DTT, 1 mM EDTA, pH 7.0). After that anthraquinone (prepared in DMSO as cosolvent, 10% v/v) was added in different concentrations followed by addition of $Na_2S_2O_4$ (74 µmol). After addition of enzyme, the reaction mixture was incubated on a rocker (gentle shaking) for 15 min at room temperature. Then, an internal standard (aloe-emodin for emodin & lunatin; 3-methoxy-chrysazin for citreorosein) was added and the product was extracted in EtOAc by vortexing and centrifugation to separate the organic and aqueous layers. The sample from organic phase was analysed using HPLC for quantitation of the product. Each reaction was performed in triplicate to validate the observed readings. The obtained peaks corresponding to internal standard and product were integrated to determine their ratio. The product concentrations in different reactions were calculated and plotted against substrate concentrations. Fitting of the data into Michaelis-Menten equation gave K_M and V_{max}. Turnover number (k_{cat}) and catalytic efficiency (η_{cat}) were also calculated based on the amount of enzyme used in the reactions.

The rate of product formation was plotted against substrate concentration and fitted using Michaelis-Menten equation to determine K_M & V_{max}:

$V_o = V_{max}[S]/(K_M+[S])$

where V_o = reaction velocity, $V_{max} = k_{cat}.[E_T]$ = maximum velocity, k_{cat} = catalyst rate constant or turnover number, $[E_T]$ = enzyme concentration, [S] = substrate concentration, K_M = Michaelis-Menten constant.

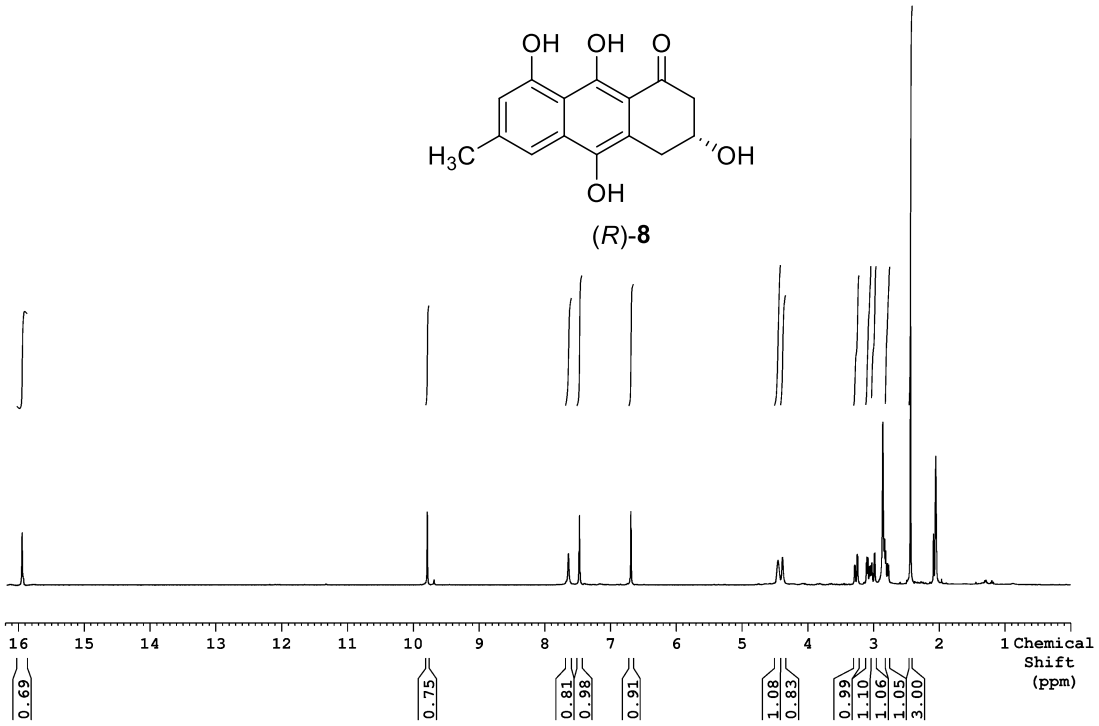
Further, catalytic efficiency ($\eta_{cat} = k_{cat}/K_M$) shows how efficiently the enzyme converts substrate into product.

Table S2: Kinetic parameter for ARti-2/ARti with anthrahydroquinones

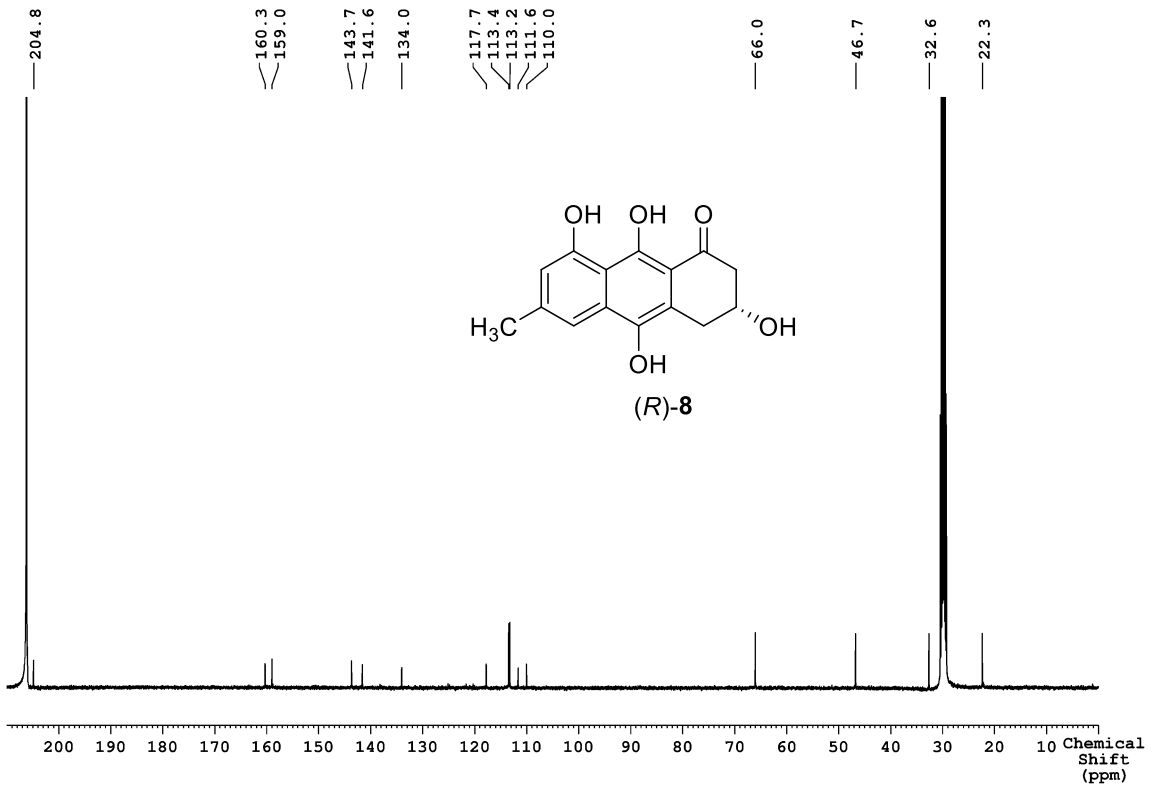
Entry	Enzyme	Substrate	K_M (M)	k_{cat} (s^{-1})	$\eta_{cat} = k_{cat}/K_M$ ($s^{-1}.M^{-1}$)
1	ARti	**10a**/**10b**	4.82×10^{-4}	1.72	3.56×10^{3}
2	ARti-2	**10a**/**10b**	5.82×10^{-4}	2.33	4.01×10^{3}
3	ARti-2	**13a**/**13b**	1.55×10^{-3}	1.05	6.81×10^{2}
4	ARti-2	**16a**/**16b**	2.08×10^{-4}	0.82	3.95×10^{3}

VII. NMR Spectra

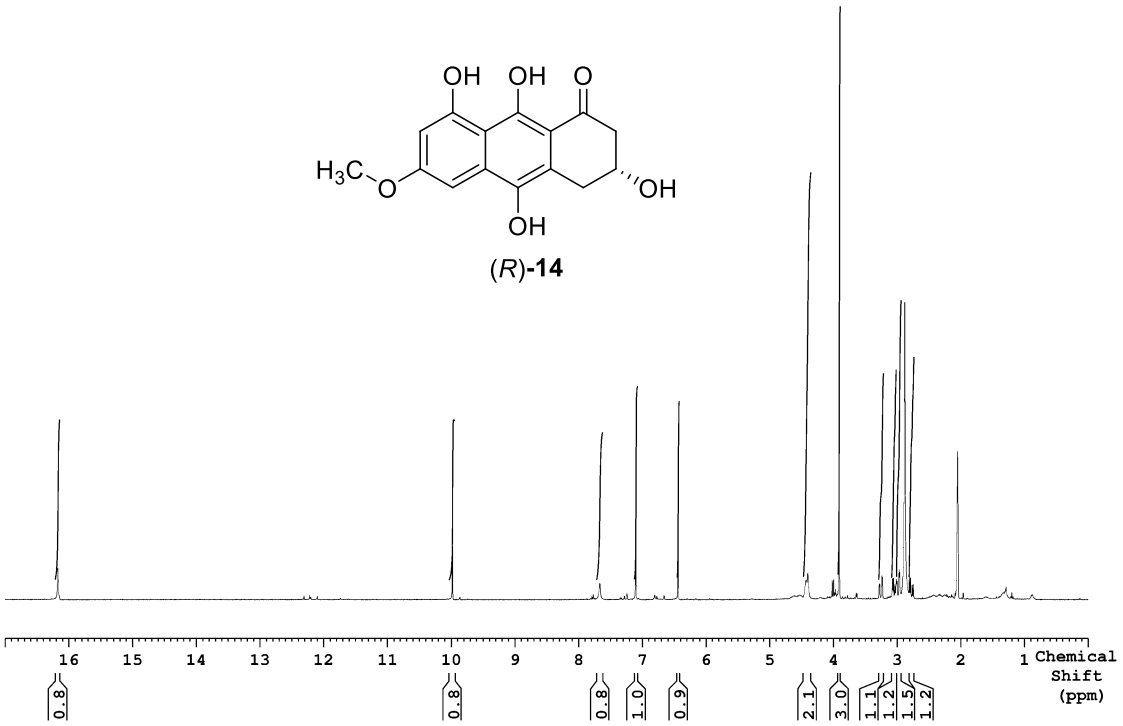
^{1}H NMR (400 MHz, acetone-*d*$_6$)



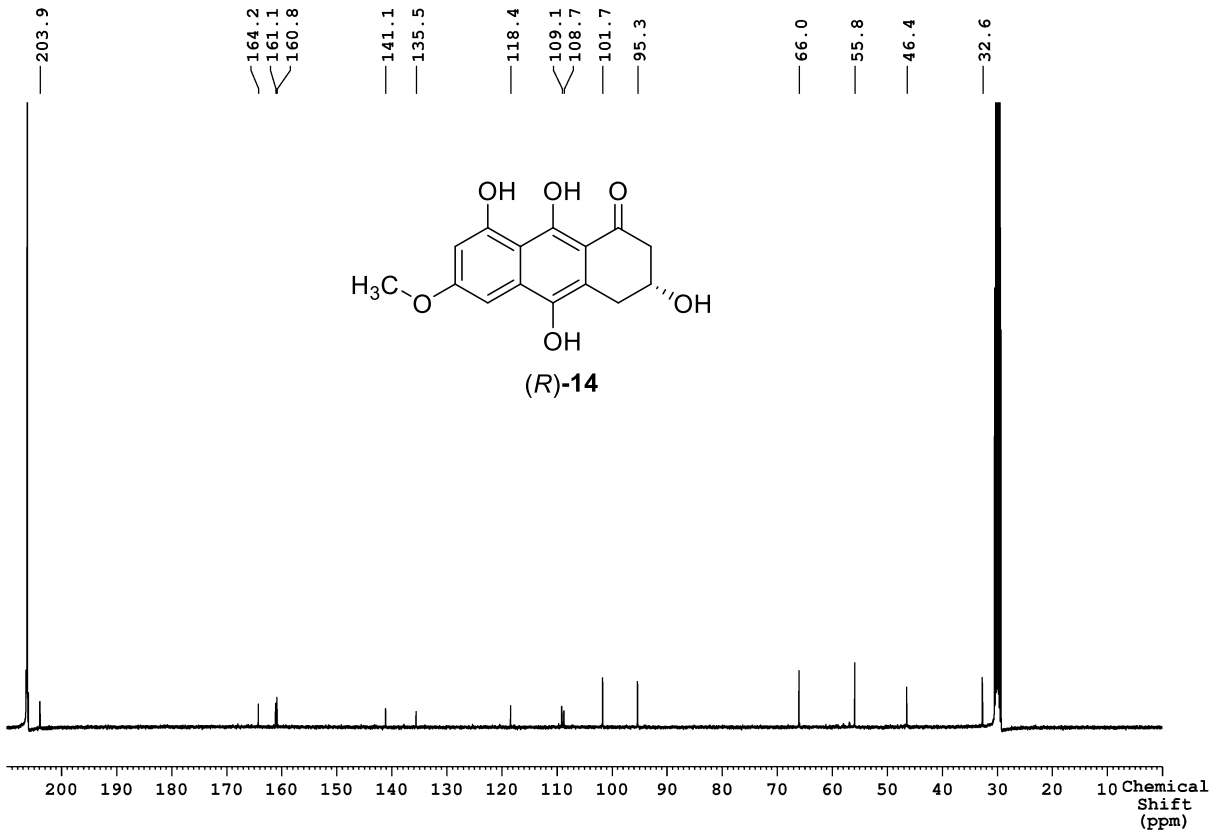
^{13}C NMR (100 MHz, acetone-*d*$_6$)



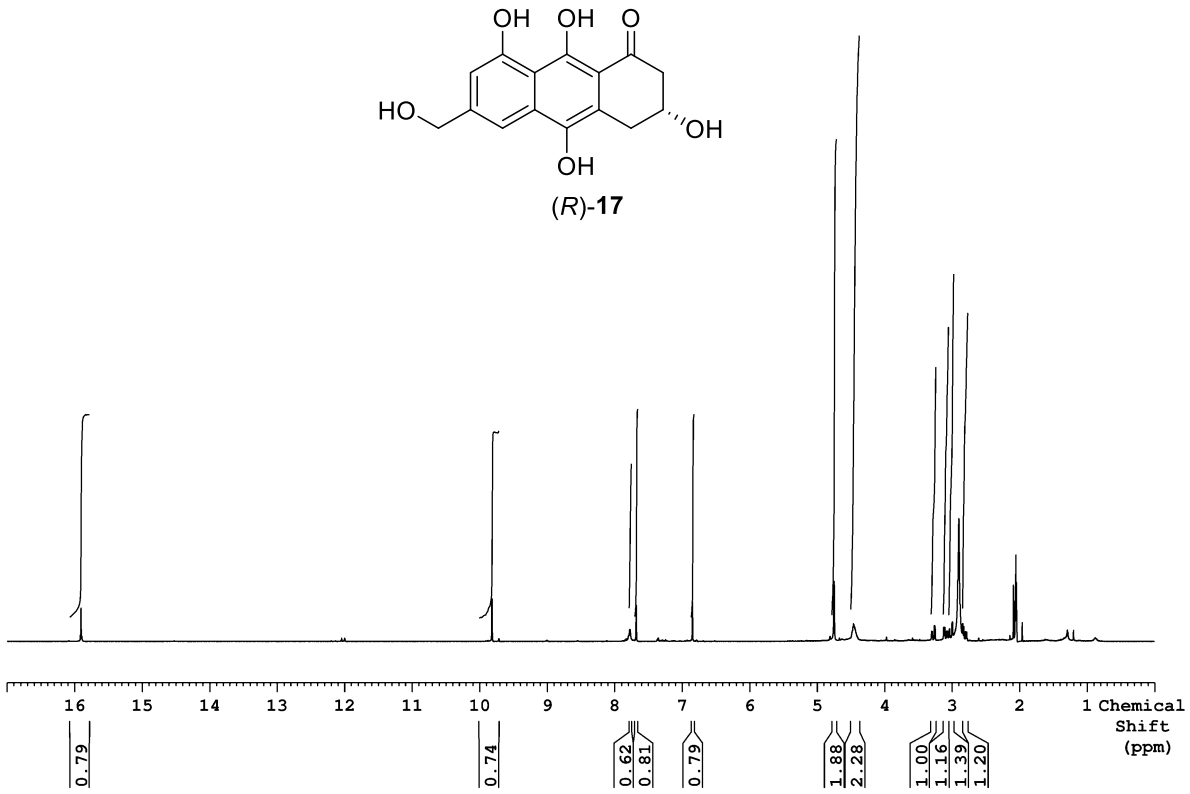
^{1}H NMR (400 MHz, acetone-d_6)



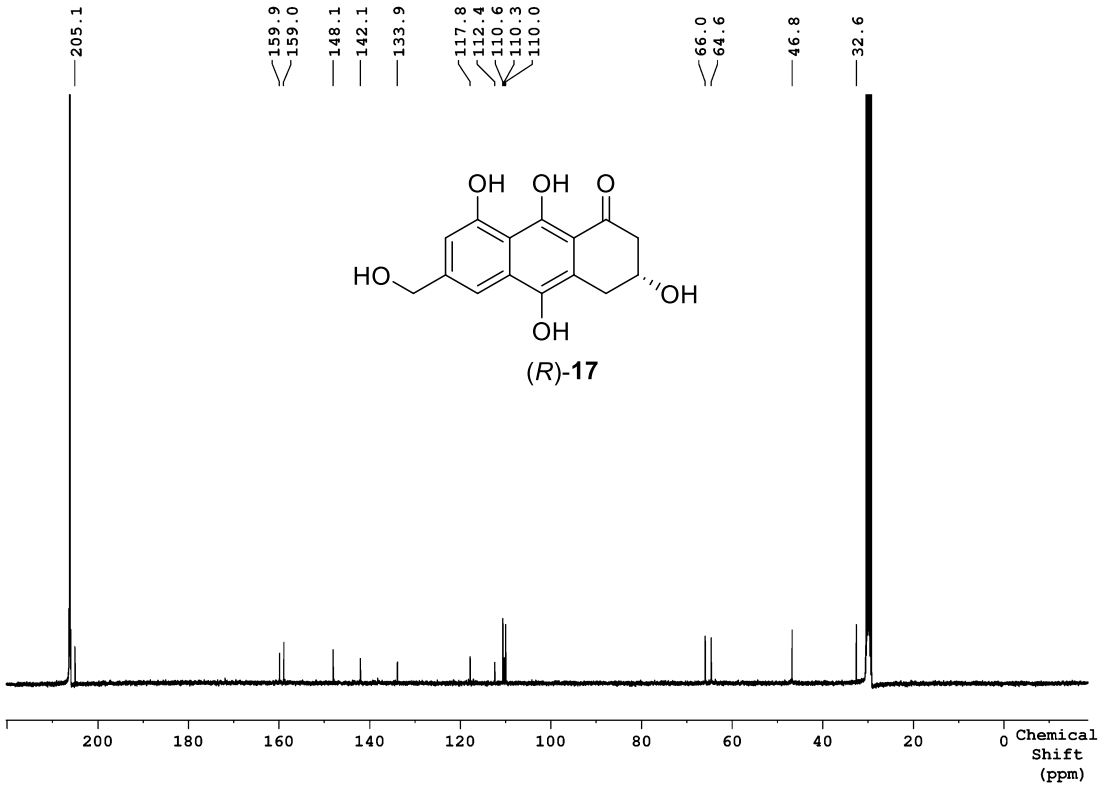
^{13}C NMR (100 MHz, acetone-d_6)



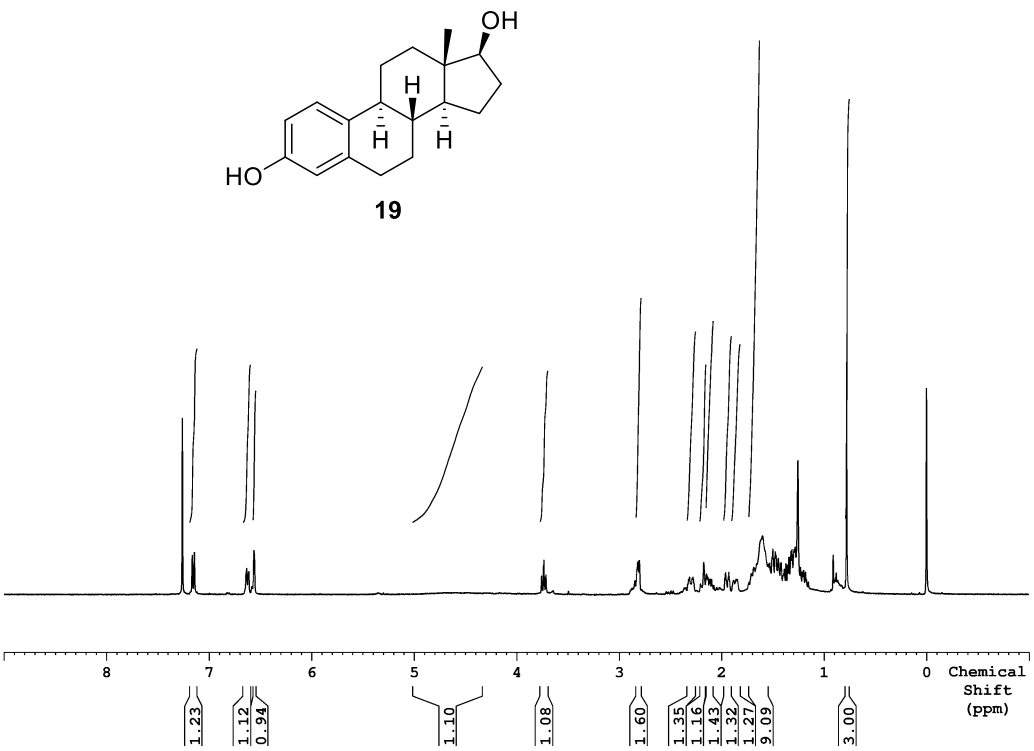
^{1}H NMR (400 MHz, acetone-d_6)



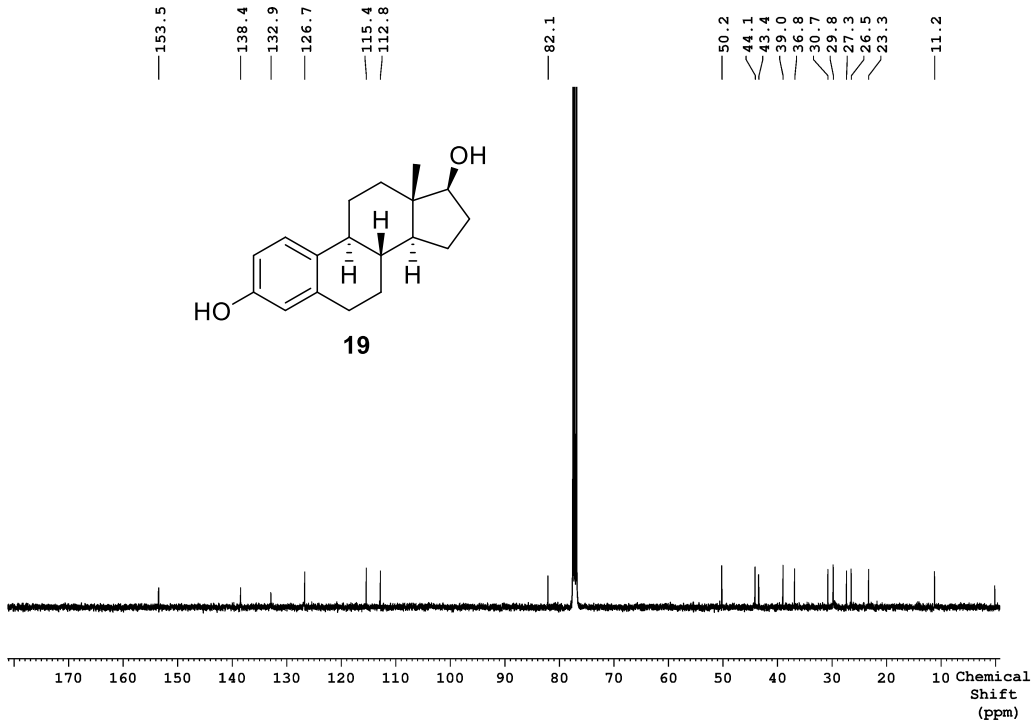
^{13}C NMR (100 MHz, acetone-d_6)



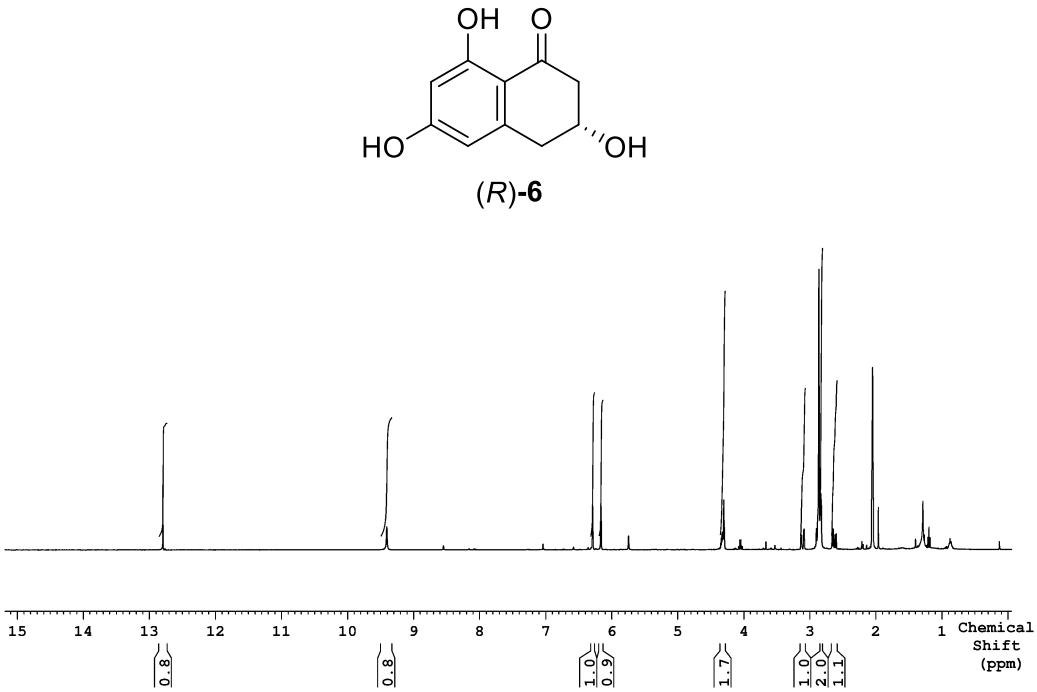
^{1}H NMR (400 MHz, $CDCl_3$)



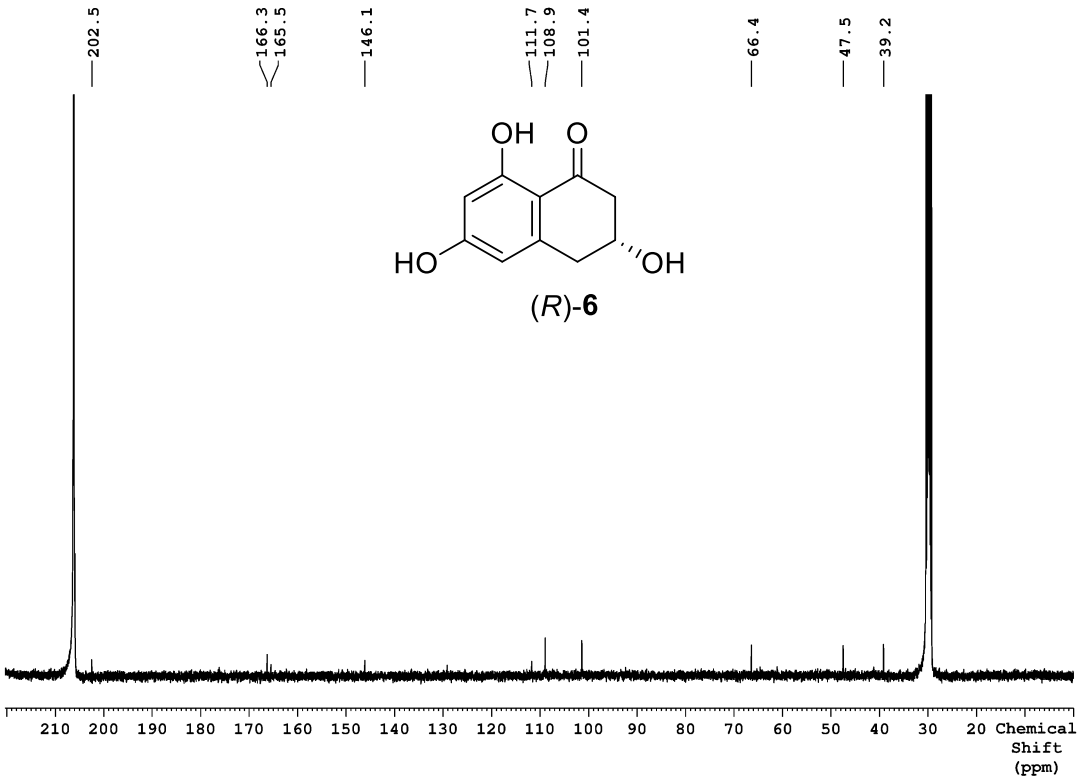
^{13}C NMR (100 MHz, $CDCl_3$)



^{1}H NMR (400 MHz, acetone-d_6)

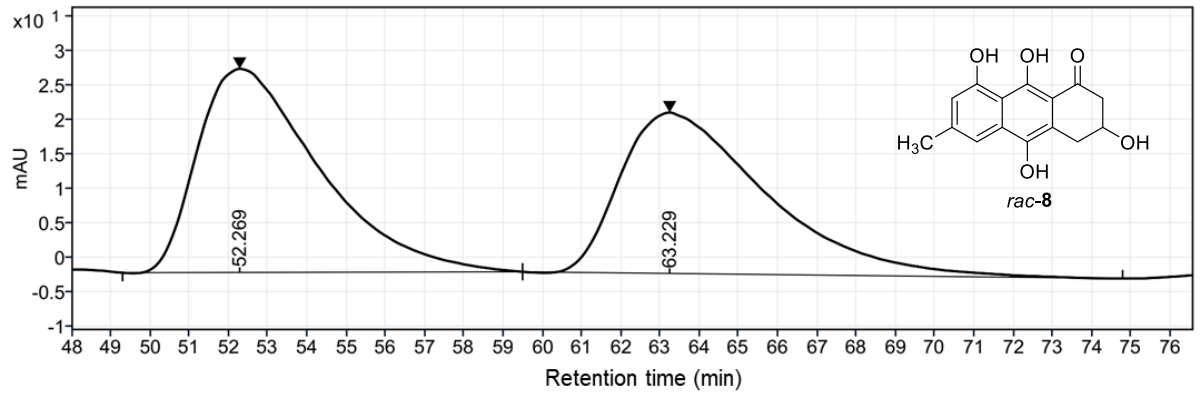


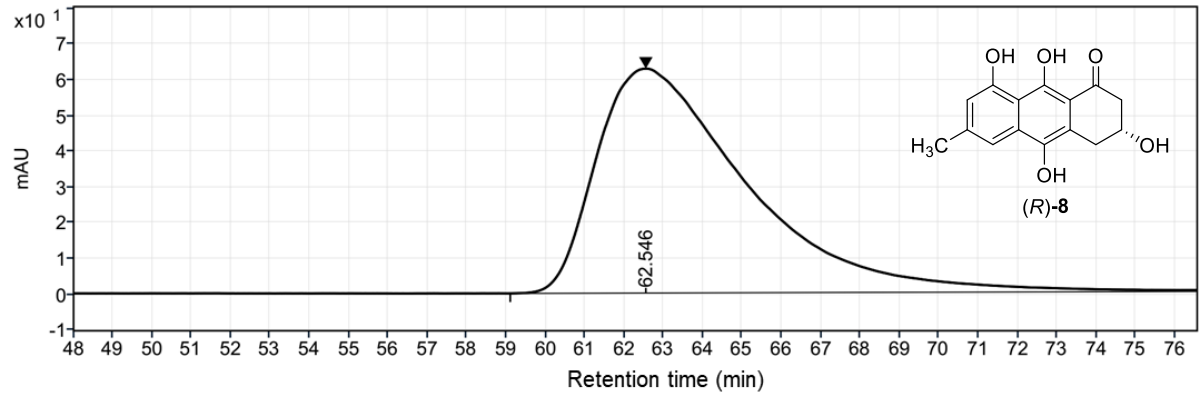
^{13}C NMR (100 MHz, acetone-d_6)

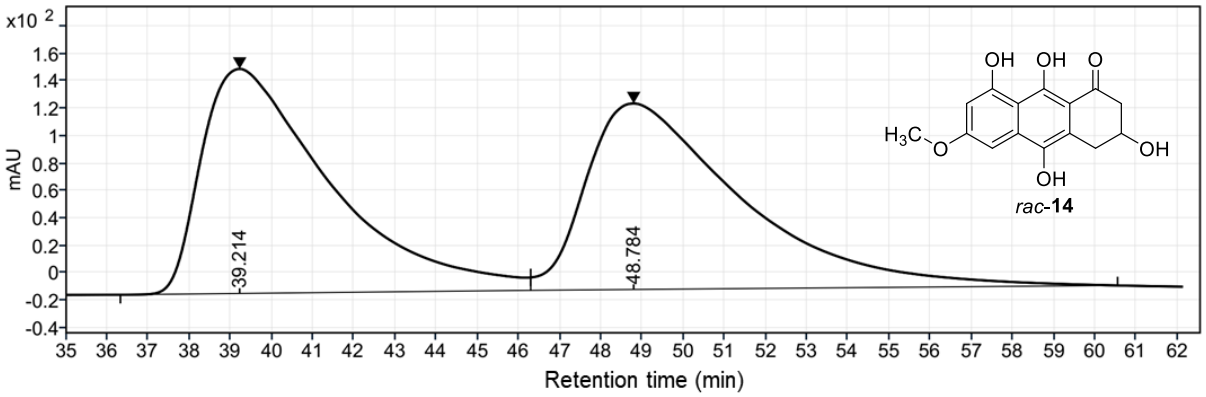


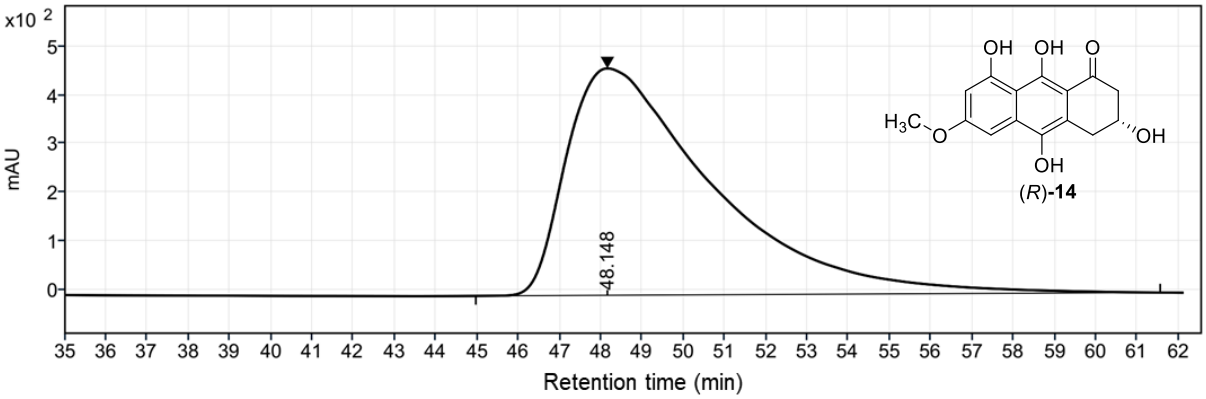
VIII. HPLC Chromatograms

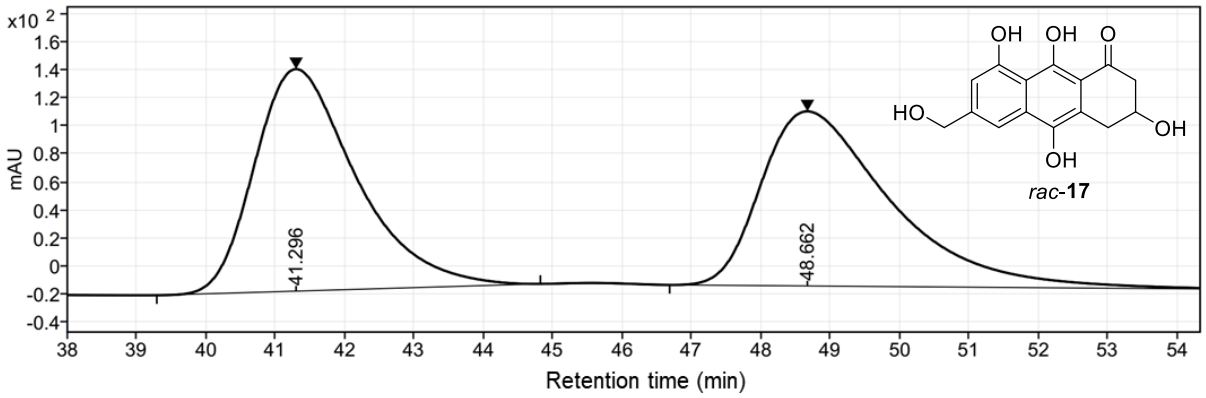
Chiral HPLC for enantiomeric excess determination

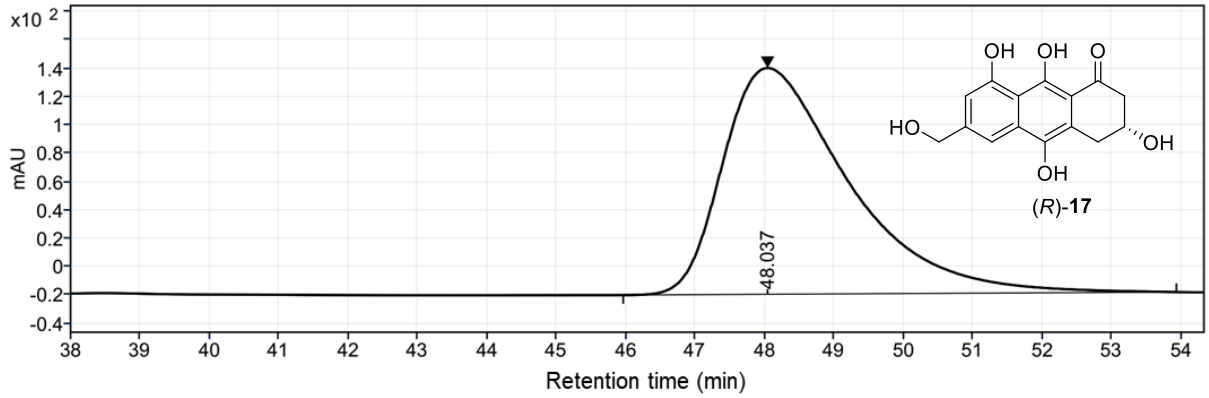


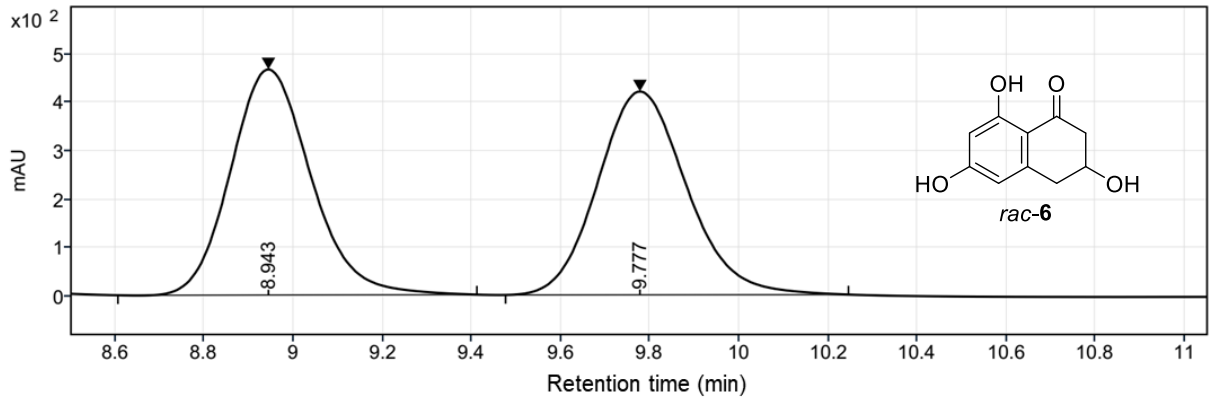


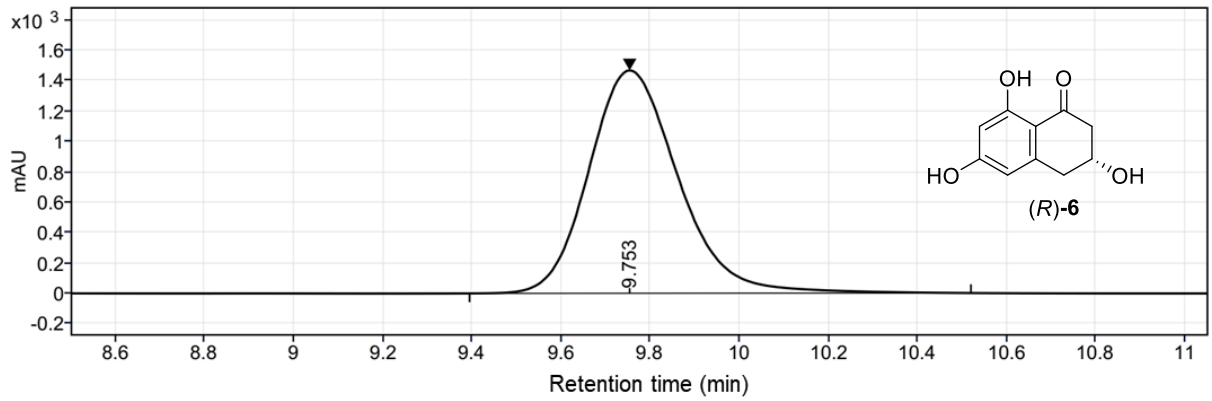




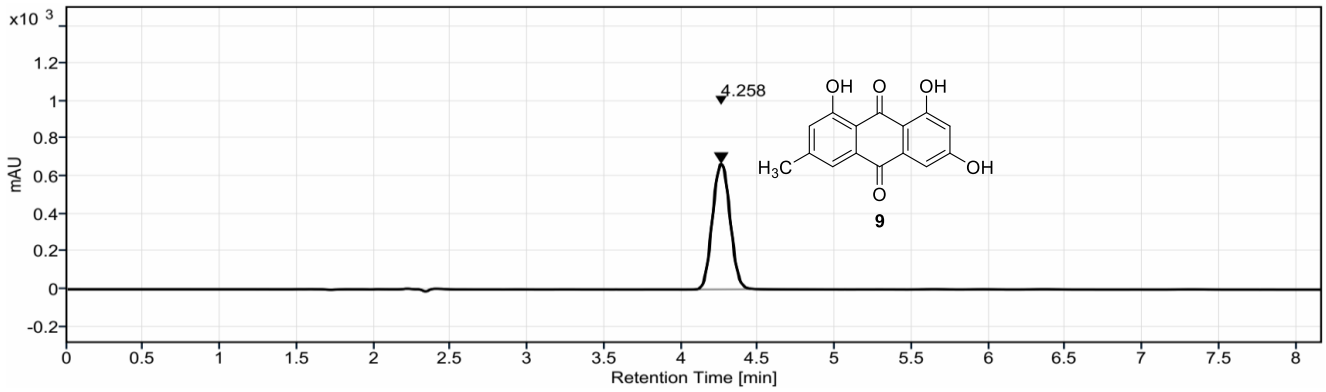

x10 2
mAU
41.296
48.662
Retention time (min)
rac-17

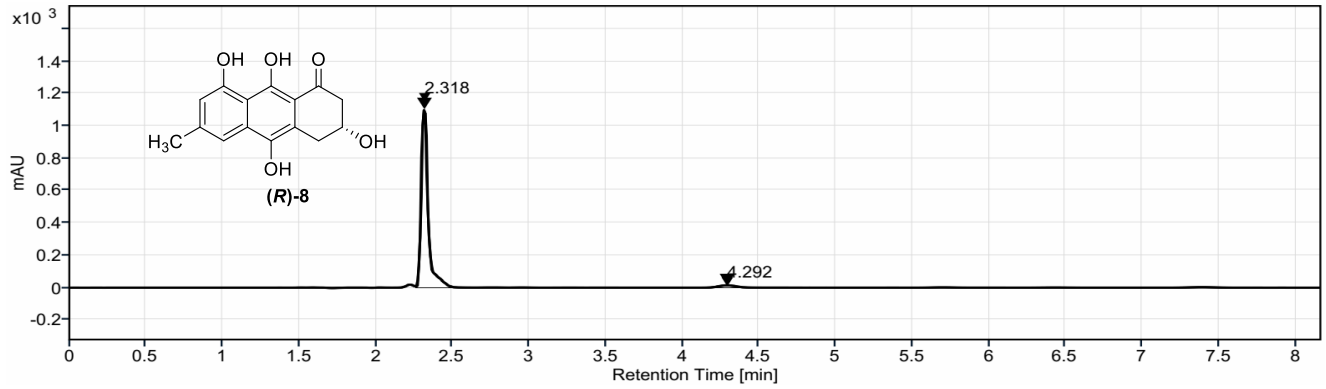

x10 2
mAU
48.037
Retention time (min)
(R)-17

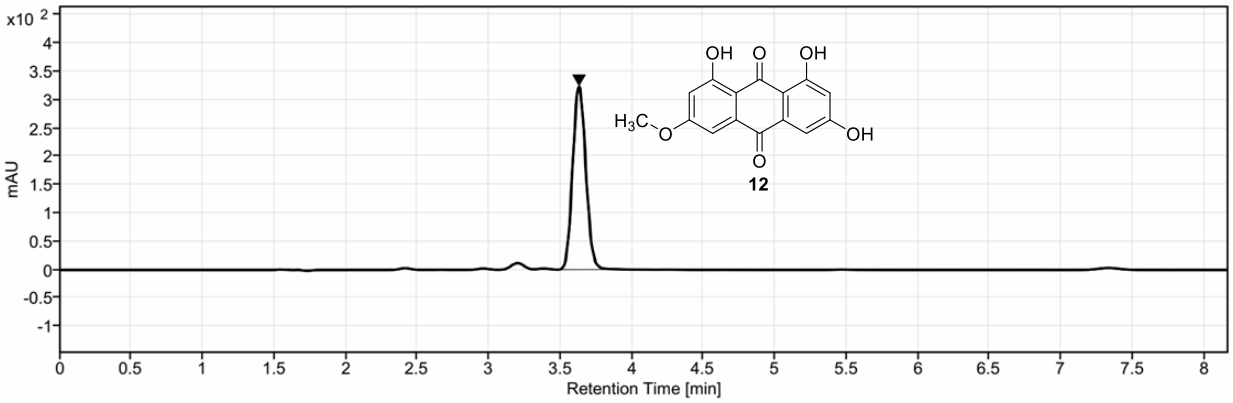

x10 2
mAU
8.943
9.777
Retention time (min)
rac-6

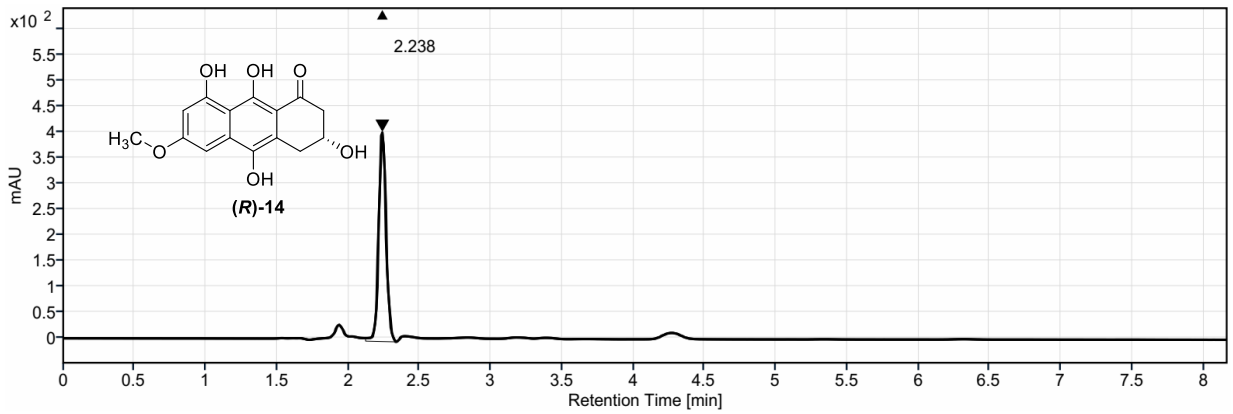

x10 3
mAU
9.753
Retention time (min)
(R)-6

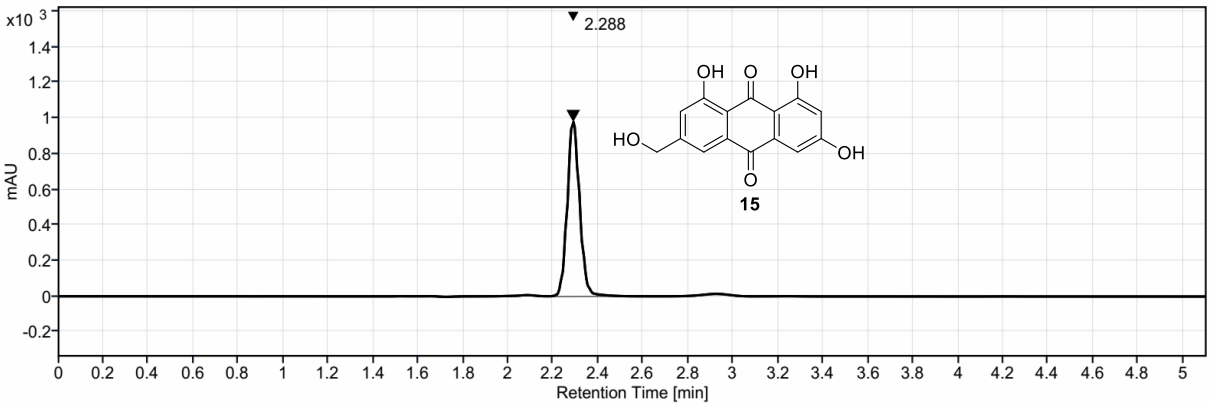
HPLC profile of ARti-2-reaction system







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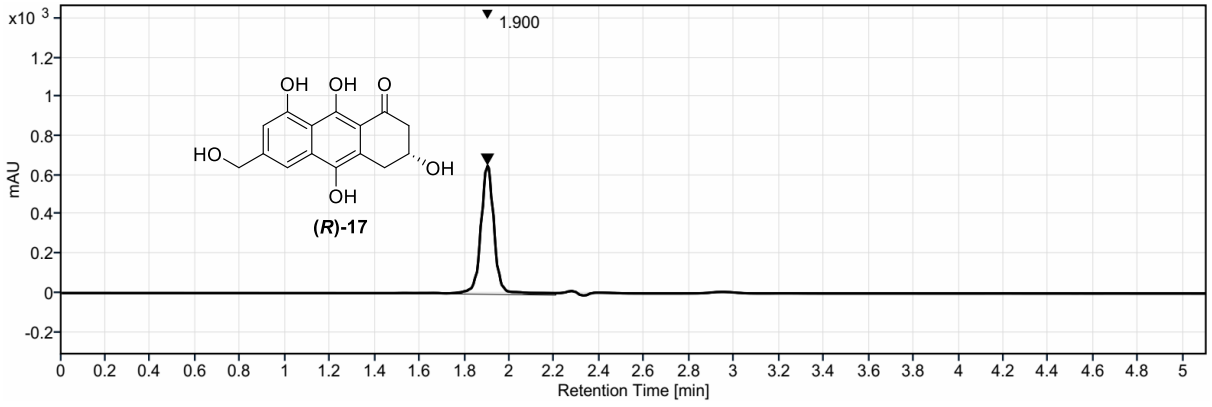
2.288
OH
O
OH
HO
OH
O
15
mAU
Retention Time [min]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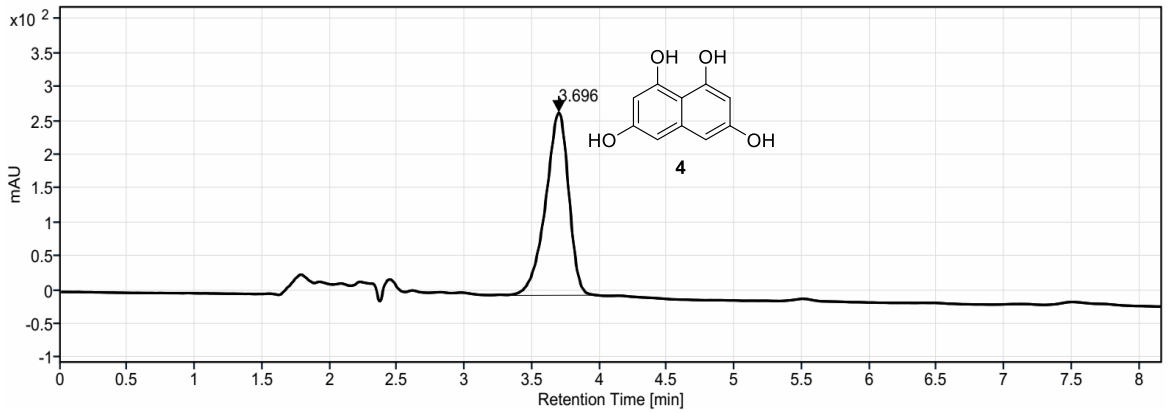
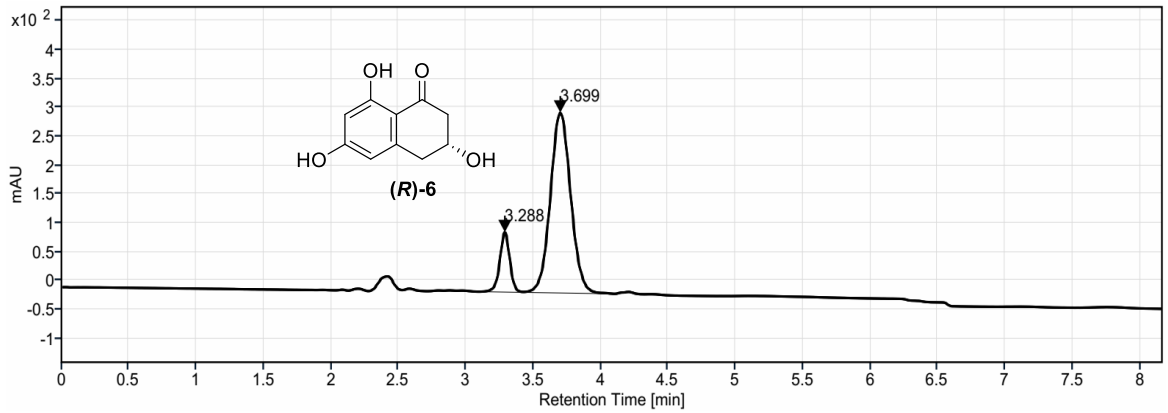
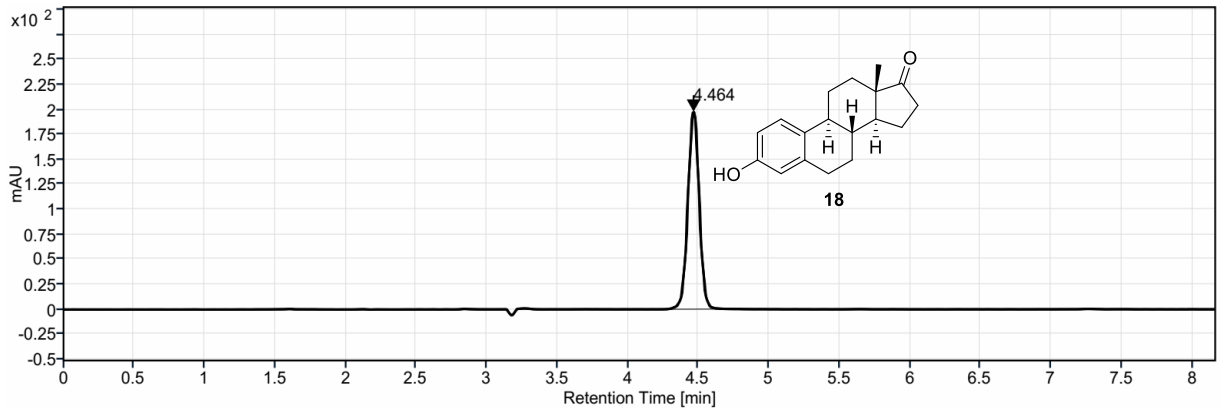
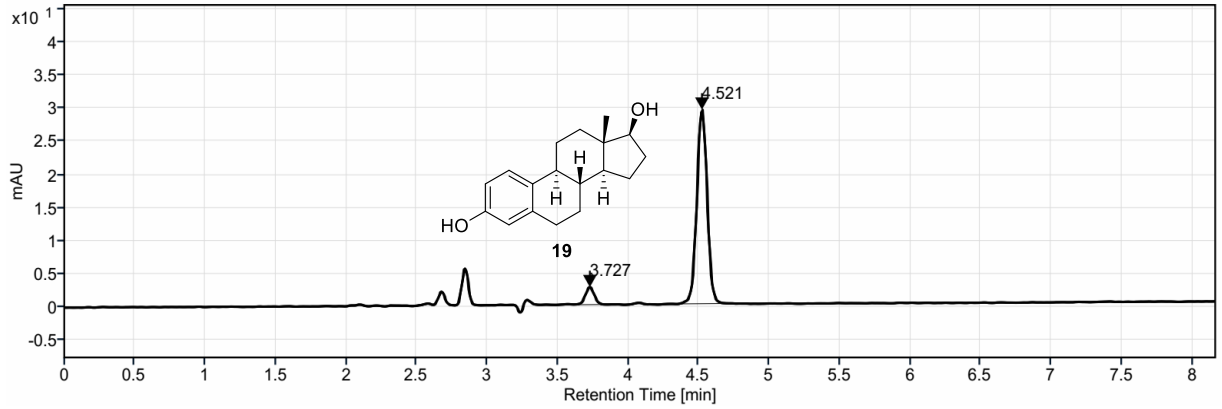
OH
OH
O
HO
OH
OH
(R)-17
mAU
Retention Time [min]


x10 2
3.696
OH
OH
HO
OH
4
mAU
Retention Time [min]


x10 2
OH
O
3.699
HO
OH
(R)-6
3.288
mAU
Retention Time [min]


x10 2
mAU
4.464
HO
18
Retention Time [min]


x10 1
mAU
4.521
OH
HO
19
3.727
Retention Time [min]

IX. References

(1) Tripathi, B.; Bhatia, R.; Pandey, A.; Gaur, J.; Chawala, G.; Walia, S.; Choi, E. H.; Attri, P. Potential Antioxidant Anthraquinones Isolated from Rheum Emodi Showing Nematicidal Activity against Meloidogyne Incognita. *J. Chem.* **2014**, *2014*, 1–9. https://doi.org/10.1155/2014/652526.

(2) Blin, K.; Wolf, T.; Chevrette, M. G.; Lu, X.; Schwalen, C. J.; Kautsar, S. A.; Suarez Duran, H. G.; de los Santos, E. L. C.; Kim, H. U.; Nave, M.; et al. AntiSMASH 4.0—Improvements in Chemistry Prediction and Gene Cluster Boundary Identification. *Nucleic Acids Res.* **2017**, *45* (W1), W36–W41. https://doi.org/10.1093/nar/gkx319.

(3) Szwalbe, A. J.; Williams, K.; Song, Z.; De Mattos-Shipley, K.; Vincent, J. L.; Bailey, A. M.; Willis, C. L.; Cox, R. J.; Simpson, T. J. Characterisation of the Biosynthetic Pathway to Agnestins A and B Reveals the Reductive Route to Chrysophanol in Fungi. *Chem. Sci.* **2019**, *10* (1), 233–238. https://doi.org/10.1039/C8SC03778G.

(4) Chojnacki, S.; Cowley, A.; Lee, J.; Foix, A.; Lopez, R. Programmatic Access to Bioinformatics Tools from EMBL-EBI Update: 2017. *Nucleic Acids Res.* **2017**, *45* (W1), W550–W553. https://doi.org/10.1093/nar/gkx273.

(5) Cassetta, A.; Stojan, J.; Krastanova, I.; Kristan, K.; Brunskole Švegelj, M.; Lamba, D.; Lanišnik Rižner, T. Structural Basis for Inhibition of 17β-Hydroxysteroid Dehydrogenases by Phytoestrogens: The Case of Fungal 17β-HSDcl. *J. Steroid Biochem. Mol. Biol.* **2017**, *171*, 80–93. https://doi.org/10.1016/j.jsbmb.2017.02.020.

(6) Wang, J.; Youkharibache, P.; Zhang, D.; Lanczycki, C. J.; Geer, R. C.; Madej, T.; Phan, L.; Ward, M.; Lu, S.; Marchler, G. H.; et al. ICn3D, a Web-Based 3D Viewer for Sharing 1D/2D/3D Representations of Biomolecular Structures. *Bioinformatics* **2020**, *36* (1), 131–135. https://doi.org/10.1093/bioinformatics/btz502.

(7) Ichinose, K.; Ebizuka, Y.; Sankawa, U. Mechanistic Studies on the Biomimetic Reduction of Tetrahydroxynaphthalene, a Key Intermediate in Melanin Biosynthesis. *Chem. Pharm. Bull. (Tokyo).* **2001**, *49* (2), 192–196. https://doi.org/10.1248/cpb.49.192.

(8) Saha, N.; Mondal, A.; Witte, K.; Singh, S. K.; Müller, M.; Husain, S. M. Monomeric Dihydroanthraquinones: A Chemoenzymatic Approach and Its (Bio)Synthetic Implications for Bisanthraquinones. *Chem. Eur. J.* **2018**, *24* (6), 1283–1286. https://doi.org/10.1002/chem.201705998.

(9) Mondal, A.; Saha, N.; Rajput, A.; Singh, S. K.; Roy, B.; Husain, S. M. Chemoenzymatic Reduction of Citreorosein and Its Implications on Aloe-Emodin and Rugulosin C (Bio)Synthesis. *Org. Biomol. Chem.* **2019**, *17* (38), 8711–8715. https://doi.org/10.1039/C9OB01690B.

(10) Guo, J.; Duclos, R. I.; Vemuri, V. K.; Makriyannis, A. The Conformations of 17β-Estradiol (E2) and 17α-Estradiol as Determined by Solution NMR. *Tetrahedron Lett.* **2010**, *51* (27), 3465–3469. https://doi.org/10.1016/j.tetlet.2010.04.077.

(11) Mondal, A.; Singh, S. K.; Saha, N.; Husain, S. M. A Biomimetic Synthesis of Racemic Dihydroanthracen-1(2 H)-Ones Using Sodium Borohydride in Water. *Eur. J. Org. Chem.* **2020**, *2020* (16), 2425–2430. https://doi.org/10.1002/ejoc.202000096.

(12) Singh, S. K.; Husain, S. M. A Redox-Based Superoxide Generation System Using Quinone/Quinone Reductase. *ChemBioChem* **2018**, *19* (15), 1657–1663. https://doi.org/10.1002/cbic.201800071.

(13) Schätzle, M. A.; Husain, S. M.; Ferlaino, S.; Müller, M. Tautomers of Anthrahydroquinones: Enzymatic Reduction and Implications for Chrysophanol, Monodictyphenone, and Related Xanthone Biosyntheses. *J. Am. Chem. Soc.* **2012**, *134* (36), 14742–14745. https://doi.org/10.1021/ja307151x.