ChemBioChem

Supporting Information

Comparing the Catalytic and Structural Characteristics of a 'Short' Unspecific Peroxygenase (UPO) Expressed in *Pichia pastoris* and *Escherichia coli*

Wendy X. Q. Robinson, Tamara Mielke, Benjamin Melling, Anibal Cuetos, Alison Parkin, William P. Unsworth, Jared Cartwright, and Gideon Grogan*

1. Target Selection

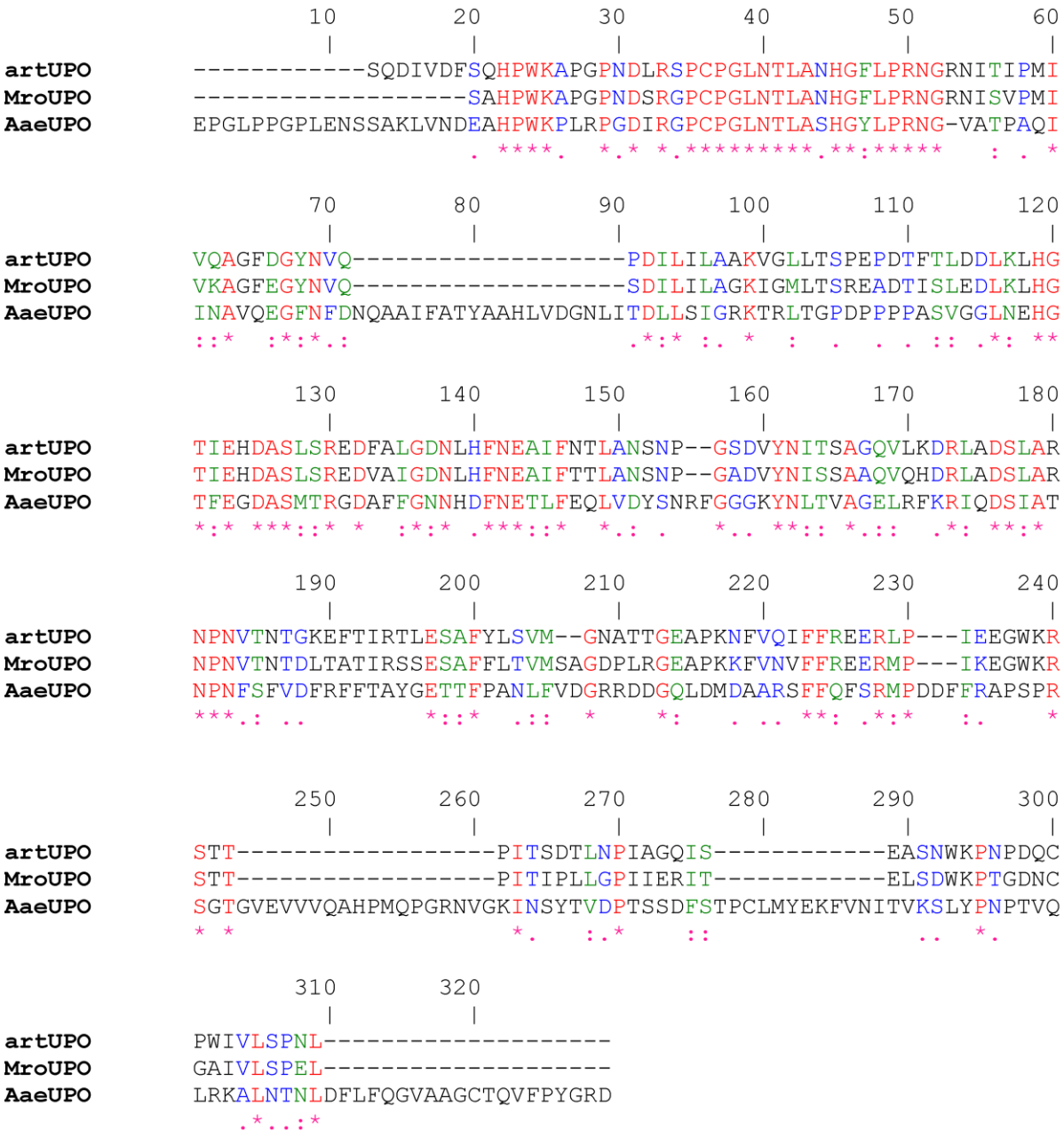

Figure S1. Sequence Alignment of artUPO, *Mro*UPO (derived from PDB deposition code 5FUJ) and *Aae*UPO PaDa-I mutant (AaeUPO, derived from PDB deposition code 6EL0).

2. Cloning, Expression and Purification of artUPO Gene in *P. pastoris* (artUPO$_{yeast}$).

>artUPO

TCCCAGGATATTGTGGACTTCTCCCAGCATCCGTGGAAGGCACCTGGACCCAACGATCTCCG
CTCCCCCTGTCCCGGACTCAACACGCTCGCGAACCACGGCTTCCTCCCTCGCAACGGTCGAA
ACATCACGATCCCGATGATCGTCCAGGCAGGCTTCGACGGCTACAACGTCCAGCCGGATATC
CTCATTTTGGCAGCCAAGGTGGGATTGCTCACCTCGCCTGAACCCGATACCTTCACCCTCGA
CGATTTGAAGTTGCATGGAACCATCGAGCATGATGCATCGTTGTCGAGGGAGGACTTCGCGC
TCGGCGATAACCTCCACTTCAACGAGGCGATCTTCAACACCCTCGCAAACTCGAACCCTGGT
TCCGATGTCTACAACATCACCTCGGCAGGACAGGTGCTCAAGGACCGCCTCGCCGACTCGCT
CGCCAGGAACCCGAACGTCACGAACACCGGCAAAGAGTTCACTATCAGGACTTTGGAGTCCG
CCTTCTATCTCTCCGTCATGGGCAACGCAACAACAGGTGAAGCACCCAAAAACTTCGTGCAG
ATTTTCTTCCGAGAGGAGCGGCTCCCCATCGAGGAGGGCTGGAAGAGGTCCACAACCCCTAT
CACGTCGGACACGTTGAACCCCATTGCAGGCCAGATTTCGGAGGCGTCGAACTGGAAGCCCA
ACCCCGACCAGTGTCCCTGGATCGTCCTCTCGCCCAACTTG

Figure S2. Gene encoding artUPO for expression in *Pichia pastoris.*

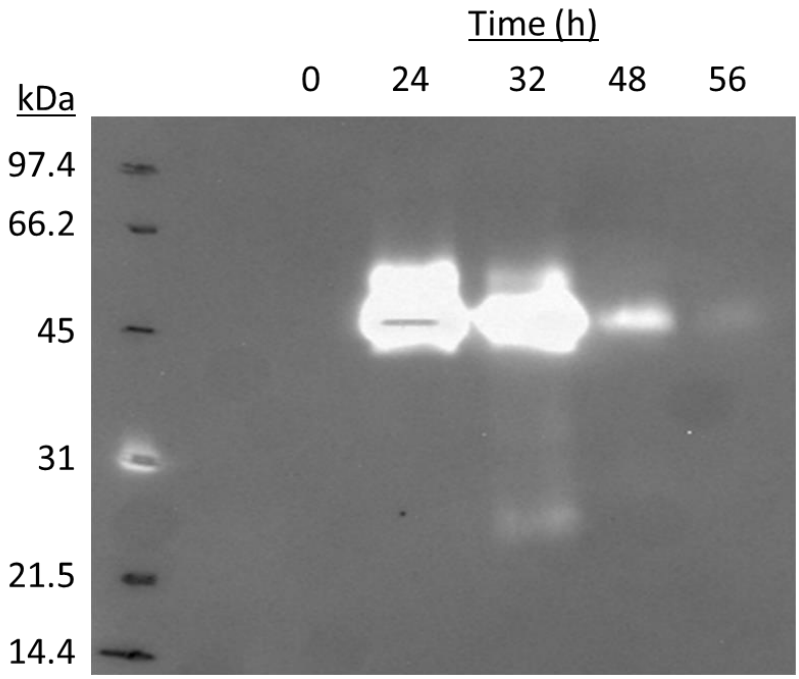

Figure S3. Expression of artUPO$_{yeast}$ in *Pichia pastoris* sampled at intervals and analysed by Western blot against anti-His as described above in section **S2**.

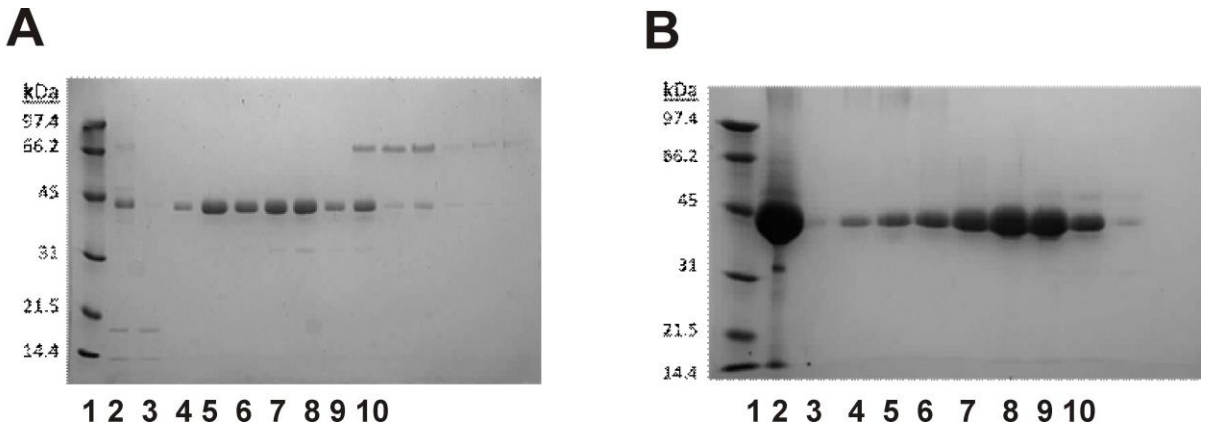

Figure S4. Purification of artUPO$_{yeast}$. **A**: SDS-PAGE analysis of fractions from MonoQ anion exchange column of artUPO. Lane 1 – low MW markers; 2: loaded protein sample; 3-10 fractions of MonoQ column; **B**: SDS-PAGE analysis of fractions from size exclusion chromatography of artUPO using a 16/600 Superdex 75 column. Lane 1 – low MW markers; 2: loaded protein sample; 3-10 fractions of SEC column.

3. Expression and Purification of artUPO Gene in *E. coli* (artUPO$_{bact}$).

The gene sequence of artUPO was codon optimised and the following sequence ordered from GeneArt inserted into pET-28a(+) (**Figure S5**).

```
ATGAGCCAGGACATCGTGGATTTTAGCCAACATCCGTGGAAAGCGCCGGGTCCGAACGACCT
GCGTAGCCCGTGCCCGGGTCTGAACACCCTGGCGAACCACGGTTTCCTGCCGCGTAACGGCC
GTAACATCACCATTCCGATGATCGTGCAGGCGGGTTTTGACGGCTACAACGTTCAACCGGAT
ATCCTGATTCTGGCGGCGAAGGTTGGTCTGCTGACCAGCCCGGAGCCGGACACCTTCACCCT
GGACGATCTGAAACTGCACGGTACCATTGAGCACGATGCGAGCCTGAGCCGTGAAGACTTTG
CGCTGGGCGATAACCTGCACTTCAACGAAGCGATCTTTAACACCCTGGCGAACAGCAACCCG
GGTAGCGATGTGTACAACATTACCAGCGCGGGCCAGGTTCTGAAGGACCGTCTGGCGGATAG
CCTGGCGCGTAACCCGAACGTGACCAACACCGGCAAAGAGTTCACCATTCGTACCCTGGAAA
GCGCGTTTTATCTGAGCGTGATGGGTAACGCGACCACCGGTGAAGCGCCGAAGAACTTTGTT
CAAATCTTCTTTCGTGAGGAACGTCTGCCGATTGAGGAAGGTTGGAAACGTAGCACCACCCC
GATCACCAGCGACACCCTGAACCCGATTGCGGGTCAGATTAGCGAAGCGAGCAACTGGAAAC
CGAACCCGGATCAATGCCCGTGGATTGTTCTGAGCCCGAACCTGTAA
```

Figure S5. Gene encoding artUPO codon optimized for expression in *E. coli.*

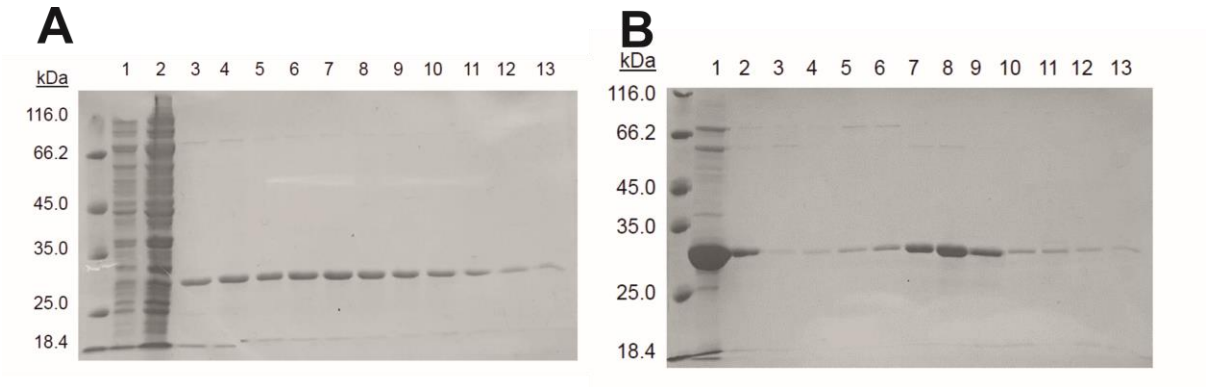

Figure S6. **A**: Fractions of NiNTA column used to isolate artUPO$_{bact}$. Lanes 1 and 2 correspond to flow through fractions; Lanes 3-13 correspond to successive fractions analysed over the A_{280} peak; **B**: Fractions of SEC column used to isolate artUPO$_{bact}$.; Lanes 1-13 correspond to successive fractions analysed over the A_{280} peak.

4. Determination of Kinetic Constants

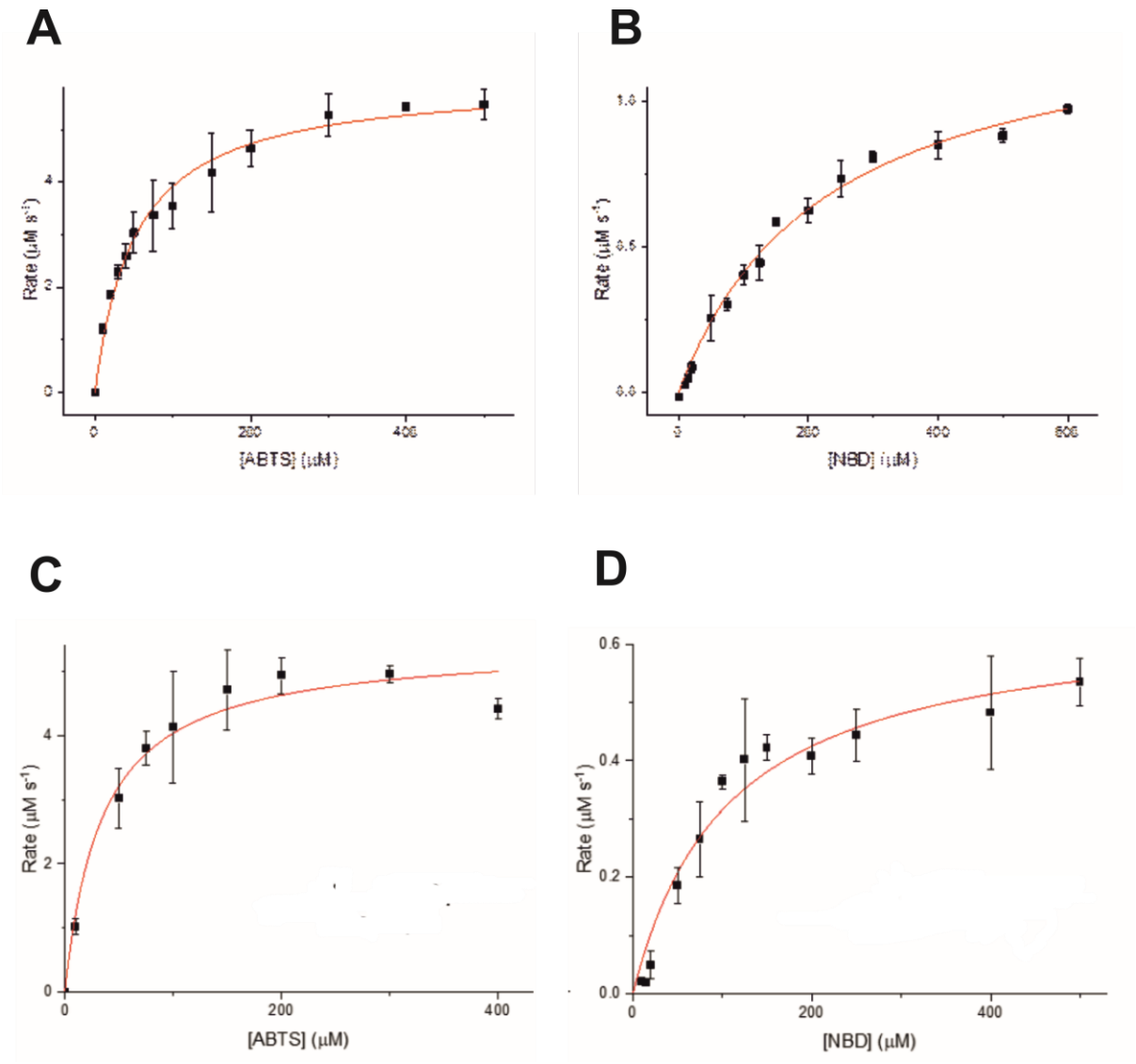

Figure S7. Determination of kinetic constants for peroxidase and peroxygenase activity of artUPO$_{yeast}$ with **A**: ABTS and **B**: NBD as substrates and artUPO$_{bact}$ with **C**: ABTS and **D**: NBD as substrates Kinetic constants can be found in manuscript **Table 1**.

5. Analytical Size Exclusion Experiments

The oligomeric nature of native artUPO$_{yeast}$ and artUPO$_{bact}$ was compared using analytical size exclusion on a Superdex™ 200 Increase 10/300 GL column using 200 µL of enzyme at 1 mg mL^{-1} in 10 mM potassium phosphate pH 7.4, containing 150 mM NaCl at a flow rate of 0.75 mL min^{-1}. Elution profiles were compared to a protein

standard mix (69385 Protein Standard Mix 15, Sigma Aldrich) run on the same column in the same conditions (**Figure S8**).

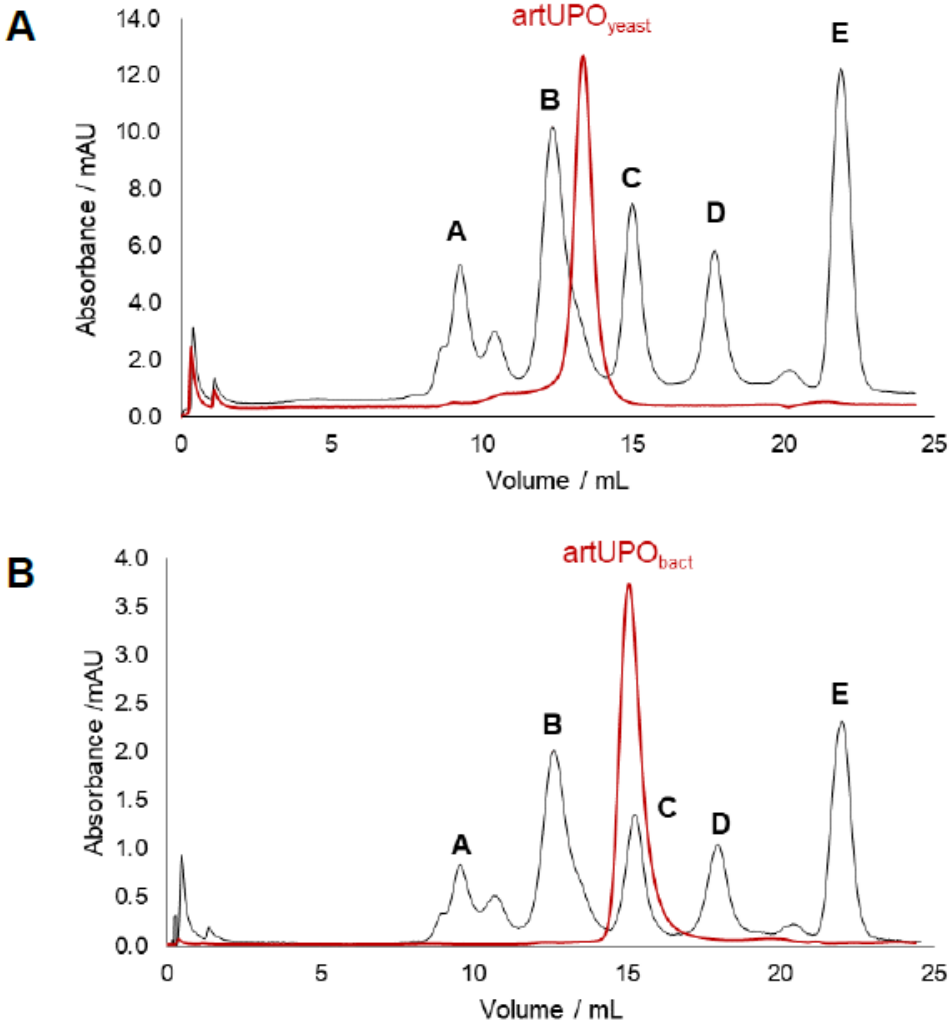

Figure S8. Analytical SEC chromatograms for **A**. artUPO$_{yeast}$ and **B**. artUPO$_{bact}$. (red traces). Standards (black trace) are: **A:** Thyroglobulin 670.0 kDa; **B**: γ-globulins from bovine blood 150.0 kDa; **C**: Albumin chicken egg grade VI (ovalbumin) 44.3 kDa; **D**: Ribonuclease A type I-A from bovine pancreas 13.7 kDa; **E**: *p*-aminobenzoic acid (pABA).

6. Crystallisation

artUPO$_{yeast}$

Purified artUPO$_{yeast}$ was concentrated to 60 mg mL^{-1} in 50 mM Tris pH 8.0, 300 mM NaCl and 10% *w/v* glycerol. Initial crystallisation screens were set up in 96 well 2 drop

plates, using a Mosquito robot, which contained either 1:1 or 1:2 ratio of protein to buffer in a sitting drop with a total volume of 300 nL. The best initial crystals were obtained from conditions containing 0.2 M calcium chloride dihydrate and 20% (w/v) PEG 3350 at pH 5.1. These gave a dataset in the $P2_12_12_1$ space group. Further crystals were obtained in conditions containing 0.15 M KSCN, 25% PEG MME 2000 with no buffer. These gave a higher resolution dataset in the $C222_1$ space group.

artUPO$_{bact}$

Purified artUPO$_{bact}$ at a concentration of 15 mg mL^{-1} in the size exclusion buffer (10 mM Tris-HCl with 300 mM NaCl, and 10% (v/v) glycerol) was similarly subjected to crystal trials. The best crystals were obtained in 0.1 M HEPES pH 7.5 with 25% (w/v) PEG 3350 and these were harvested without further optimisation for data collection.

7. Data collection, structure solution and refinement

Table S1. Data Collection and Refinement Statistics for artUPO. Numbers in brackets refer to data for highest resolution shells.

	artUPO$_{yeast}$; *P*2$_1$2$_1$2$_1$	**artUPO$_{yeast}$; *C*222$_1$**	**artUPO$_{bact}$**
Beamline	Diamond I03	Diamond I04-1	Diamond I03
Wavelength (Å)	0.976254	0.915870	0.976230
Resolution (Å)	50.57-2.01 (2.06-2.01)	77.27-1.21 (1.23-1.21)	39.34-2.09 (2.15-2.09)
Space Group	*P* 2$_1$2$_1$2$_1$	*C* 2 2 2$_1$	*P* 2$_1$2$_1$2$_1$
Unit cell (Å)	a = 50.34; b = 75.82; c = 151.70 $\alpha = \beta = \gamma = 90.00$	A = 50.85; b = 74.17; c = 154.54 $\alpha = \beta = \gamma = 90.00$	A = 46.03; b = 58.81; c = 151.46 $\alpha = \beta = \gamma = 90.00$
No. of molecules in the asymmetric unit	2	1	2
Unique reflections	39554 (2861)	89172 (4348)	25200 (1910)
Completeness (%)	99.9 (99.9)	99.9 (100.0)	100.0 (100.0)
R_{merge} (%)	0.08 (0.50)	0.04 (0.43)	0.12 (0.77)
$R_{p.i.m.}$	0.04 (0.28)	0.02 (0.28)	0.05 (0.32)
Multiplicity	8.0 (7.8)	7.7 (6.3)	11.4 (12.7)
$\langle I/\sigma(I) \rangle$	16.5 (4.4)	23.6 (3.8)	12.8 (3.1)
Overall *B* factor from Wilson plot (Å^2)	20	11	28
$CC_{1/2}$	1.00 (0.96)	1.00 (0.92)	1.00 (0.94)
R_{cryst}/ R_{free} (%)	19.0/22.6	15.0/16.6	18.9/23.7
r.m.s.d 1-2 bonds (Å)	0.009	0.019	0.008
r.m.s.d 1-3 angles (°)	1.62	2.18	1.51
Avge main chain B (Å^2)	29	13	32
Avge side chain B (Å^2)	32	16	34
Avge water B (Å^2)	34	17	31

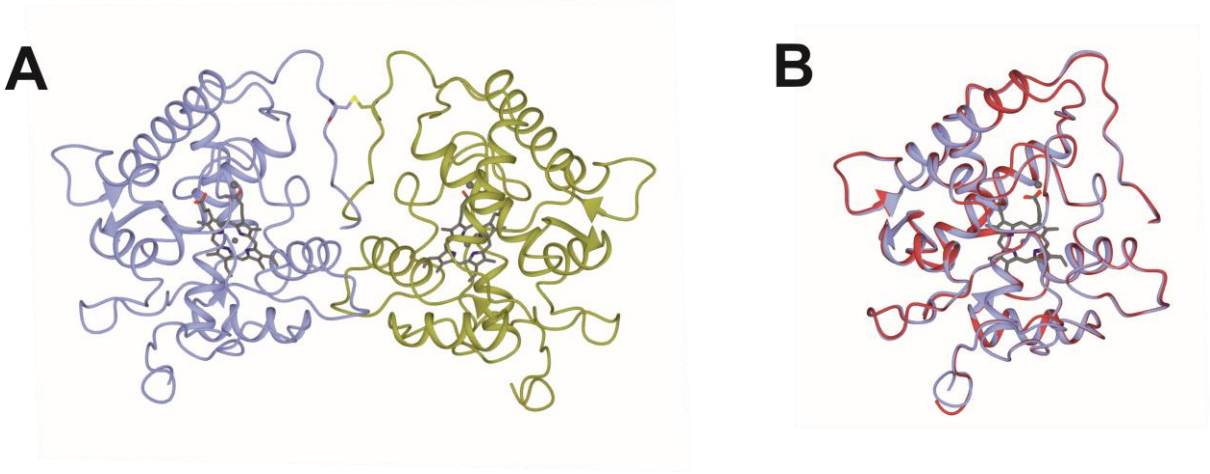

Figure S9. **A**: Structure of artUPO$_{bact}$ dimer with monomers shown in blue and gold; **B**: Superimposition of monomers of artUPO$_{bact}$ and artUPO$_{yeast}$ shown in blue and red respectively.

8. NanoDSF (Nano Differential Scanning Fluorimetry)

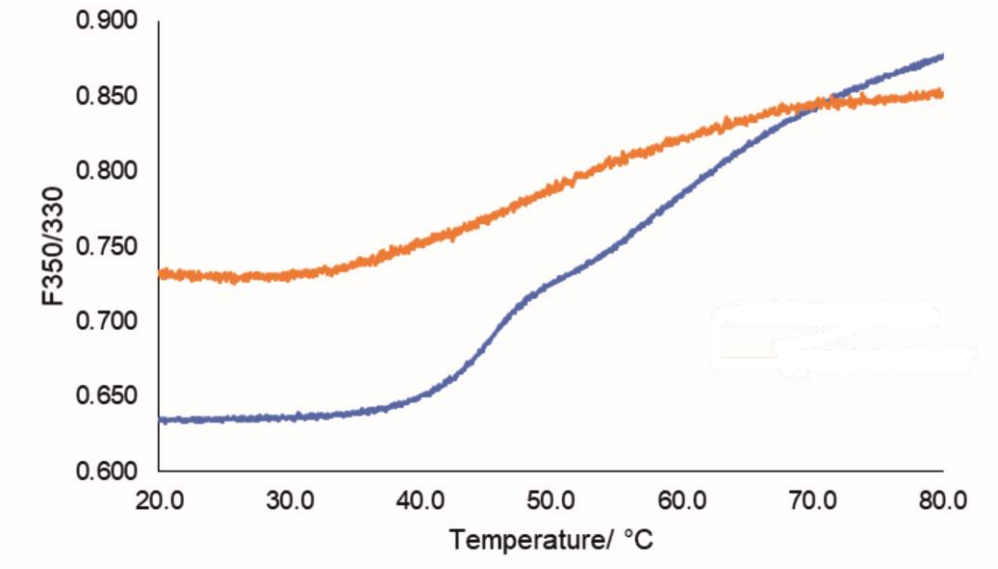

Figure S10. Nano-DSF analysis of artUPO$_{yeast}$ (blue line) and artUPO$_{bact}$ (orange). The ratio of the fluorescence at 350 nm and 330 nm was measured against a temperature gradient of 20–80 °C using 2 mg mL^{-1} of each enzyme.

9. GC Analysis

Table S2. Conditions for chiral separations of oxygenation products.

Compound	Column	Conditions	Retention Times of Enantiomers (min)
15	Betadex 120	120 °C isothermal	6.65 and 7.07
16	Betadex 120	120 °C isothermal	12.00 and 13.24
17	Betadex 120	120 °C isothermal	10.40 and 10.76
18	Betadex 120	120 °C isothermal	9.94 and 10.40
19	Betadex 120	120 °C isothermal	7.35 and 7.71
20	Betadex 120	120 °C isothermal	22.18 and 23.75
21	Betadex 120	120 °C isothermal	28.36 and 27.40
26	BGB 175	180 °C isothermal	5.33 and 5.86
27	BGB 175	180 °C isothermal	5.37 and 5.65 (4.21 and 4.42)*
28	BGB 175	180 °C isothermal	4.29 and 4.73
29	BGB 175	180 °C isothermal	10.54 and 11.32

*****The determination of the *ee* of **27** using artUPO$_{bact}$ was conducted with a new BGB175 column, with adjusted retention times for enantiomers using the same conditions, as indicated.

10. Chiral GC Traces

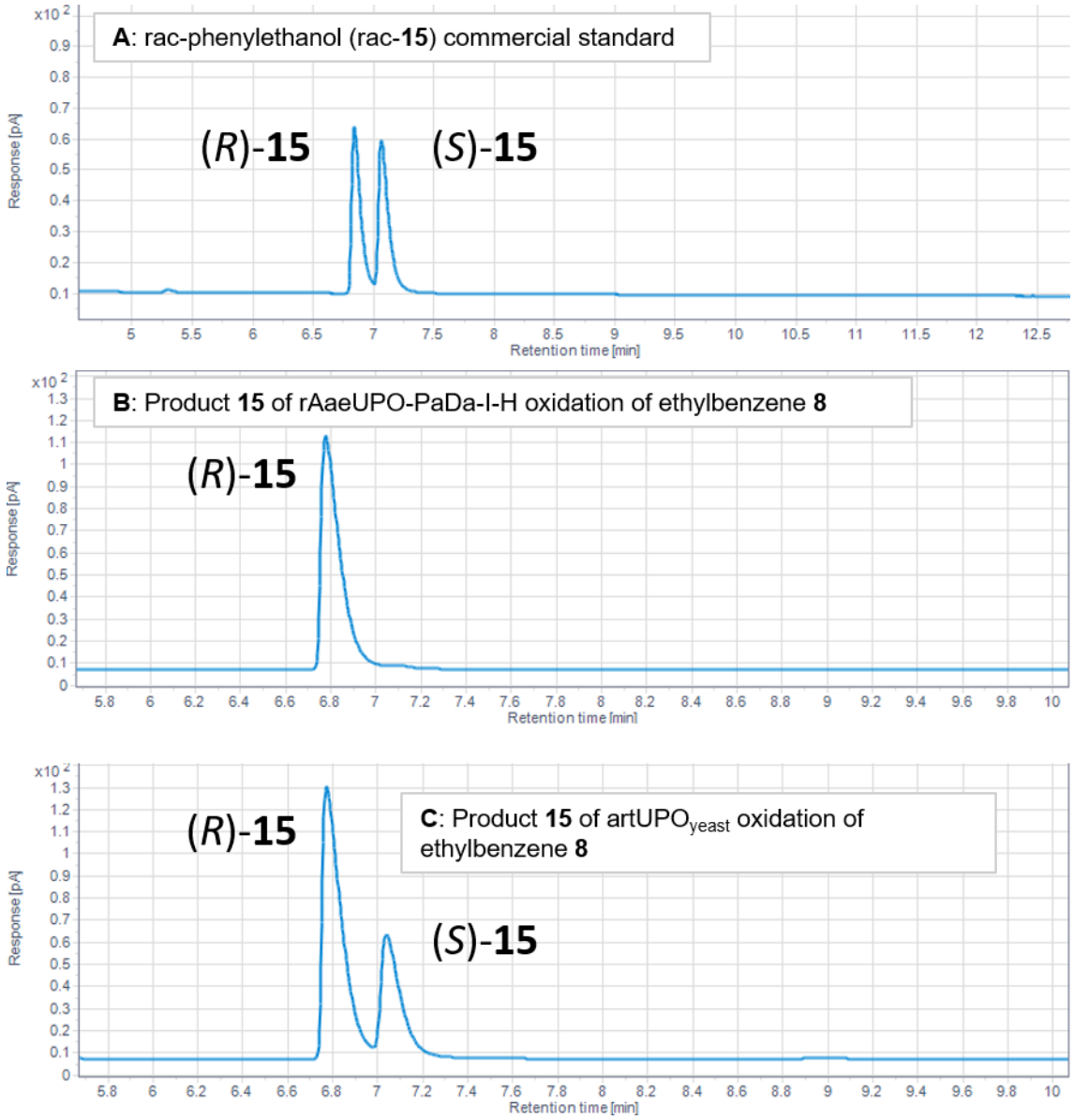

Figure S11. Chiral separation of enantiomers of **15**.

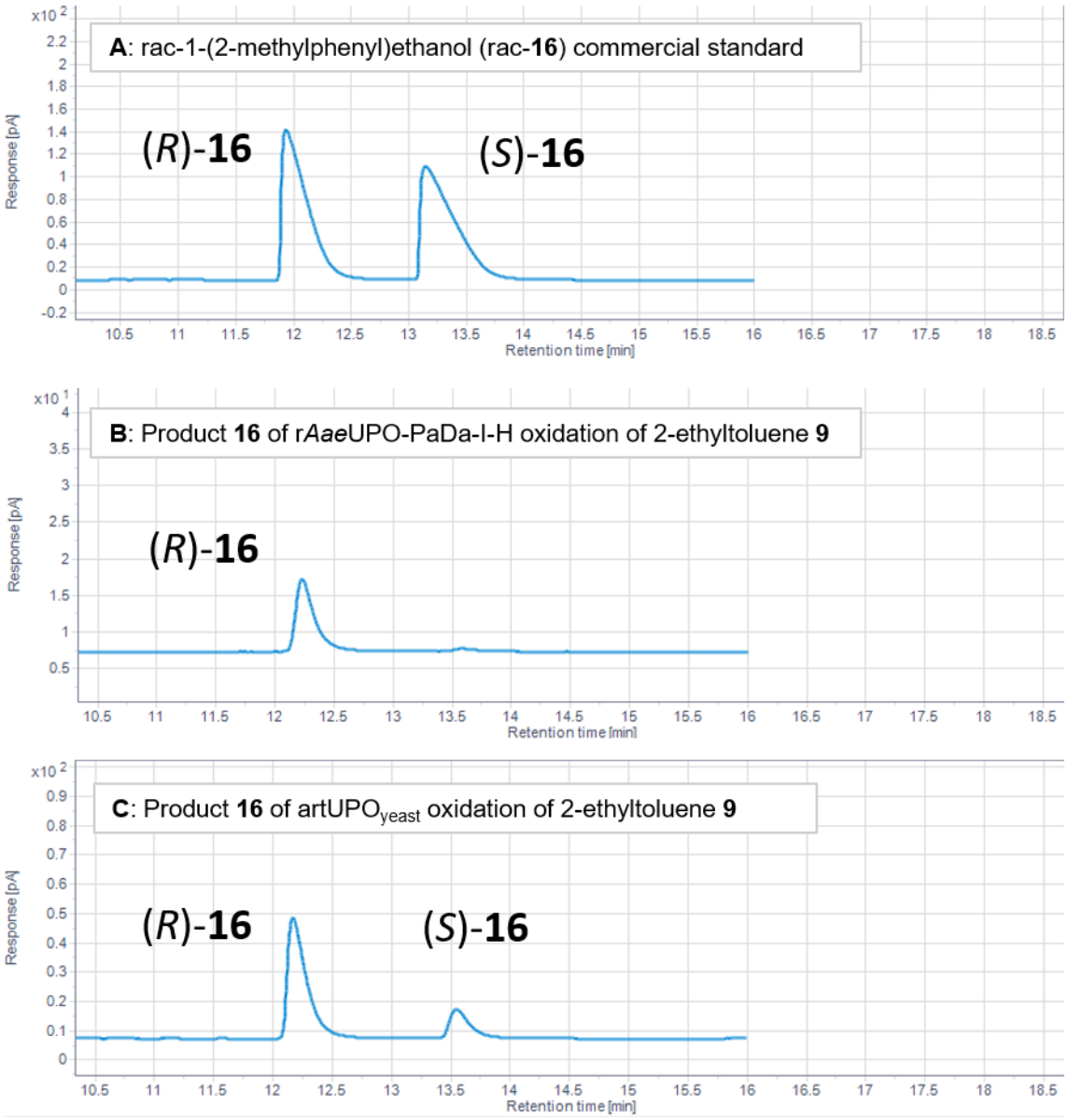

Figure S12. Chiral separation of enantiomers of **16**.

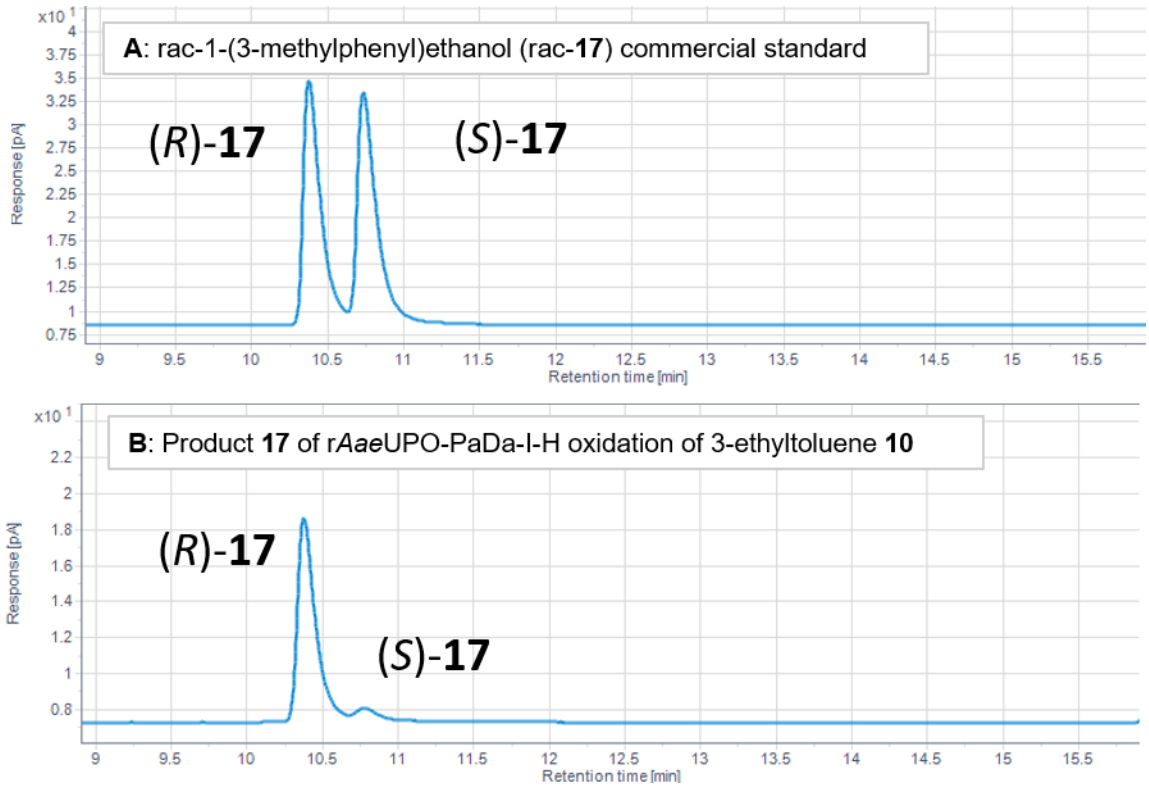

Figure S13. Chiral separation of enantiomers of **17**.

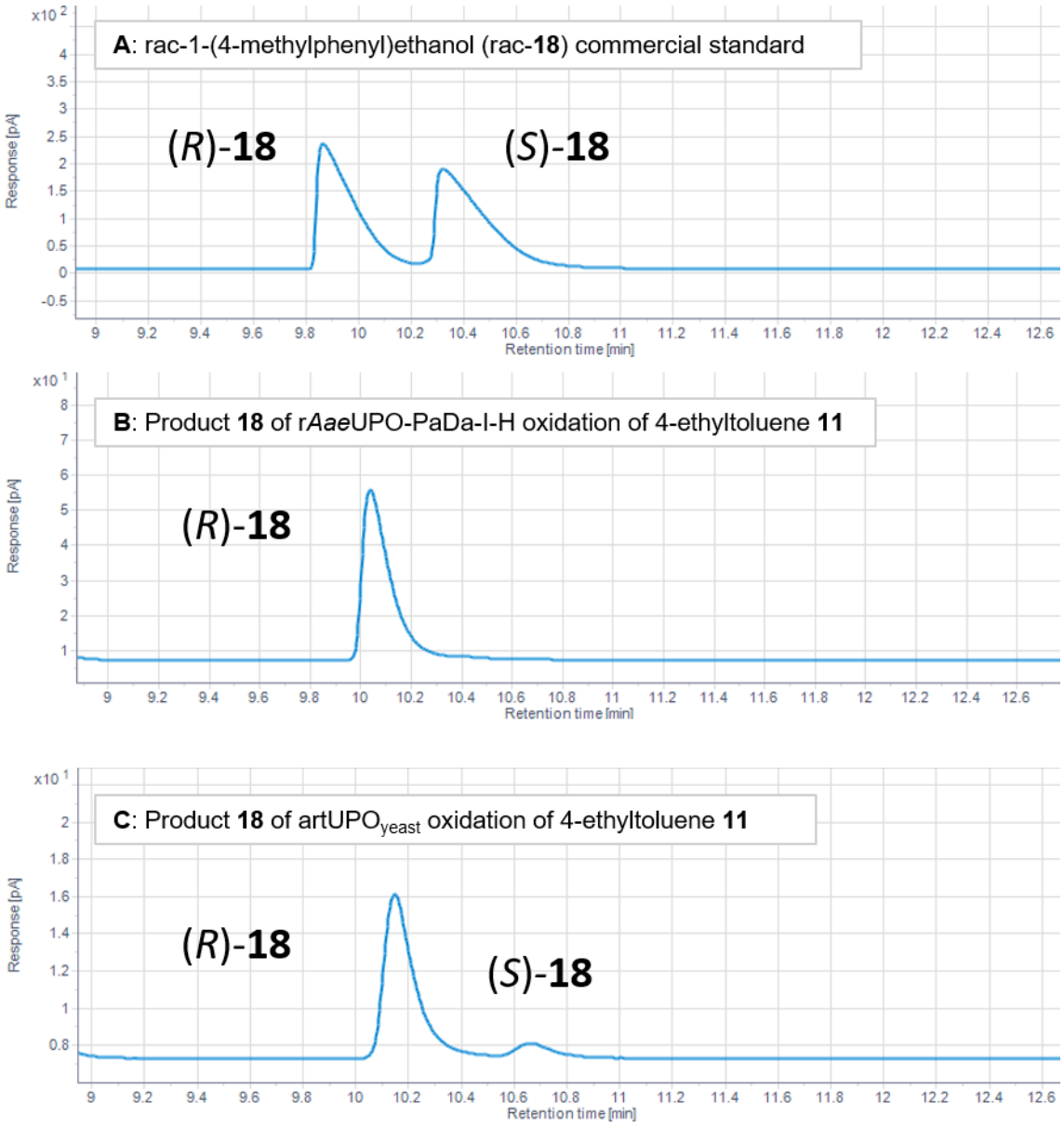

Figure S14. Chiral separation of enantiomers of **18**.

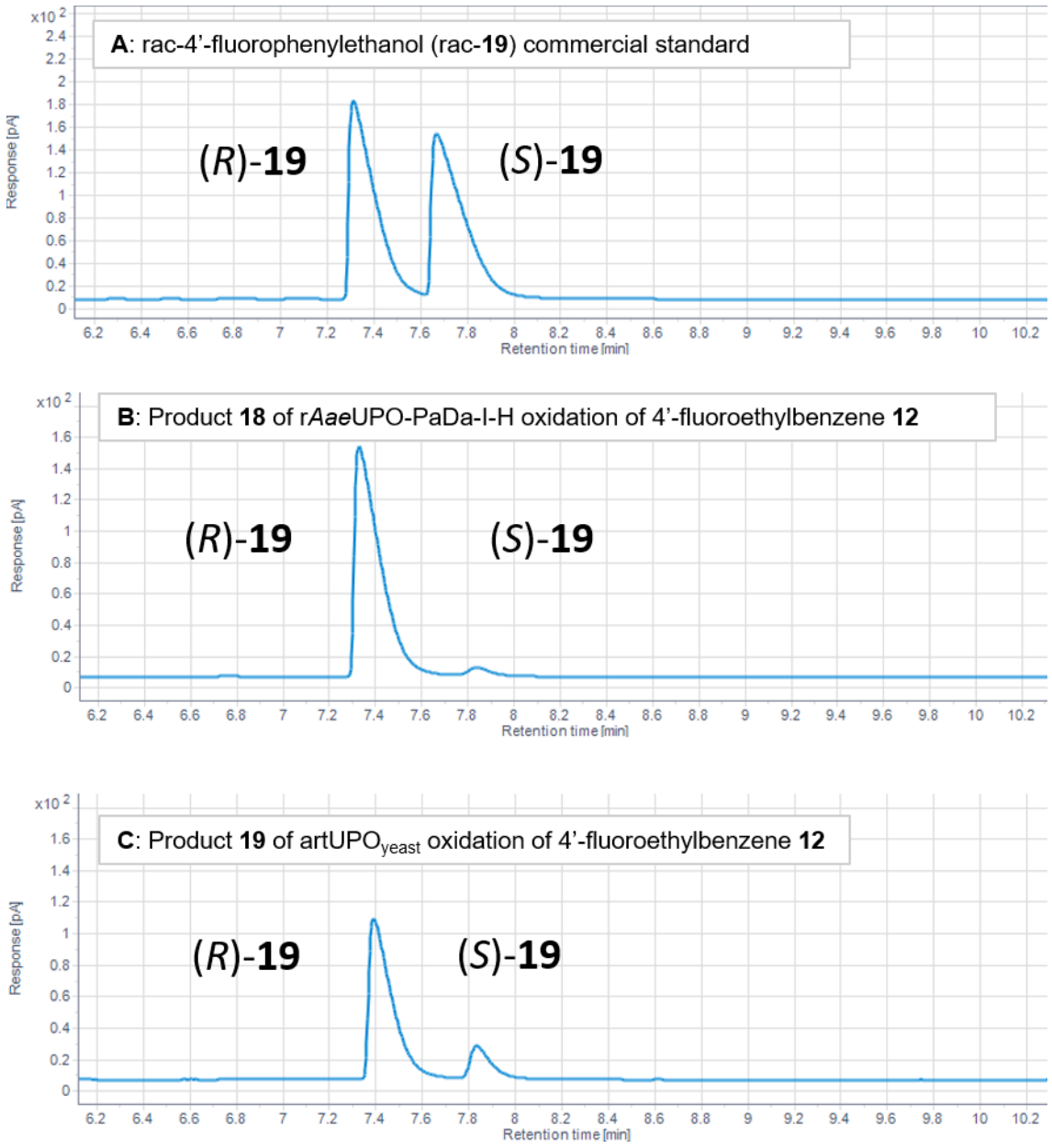

Figure S15. Chiral separation of enantiomers of **19**.

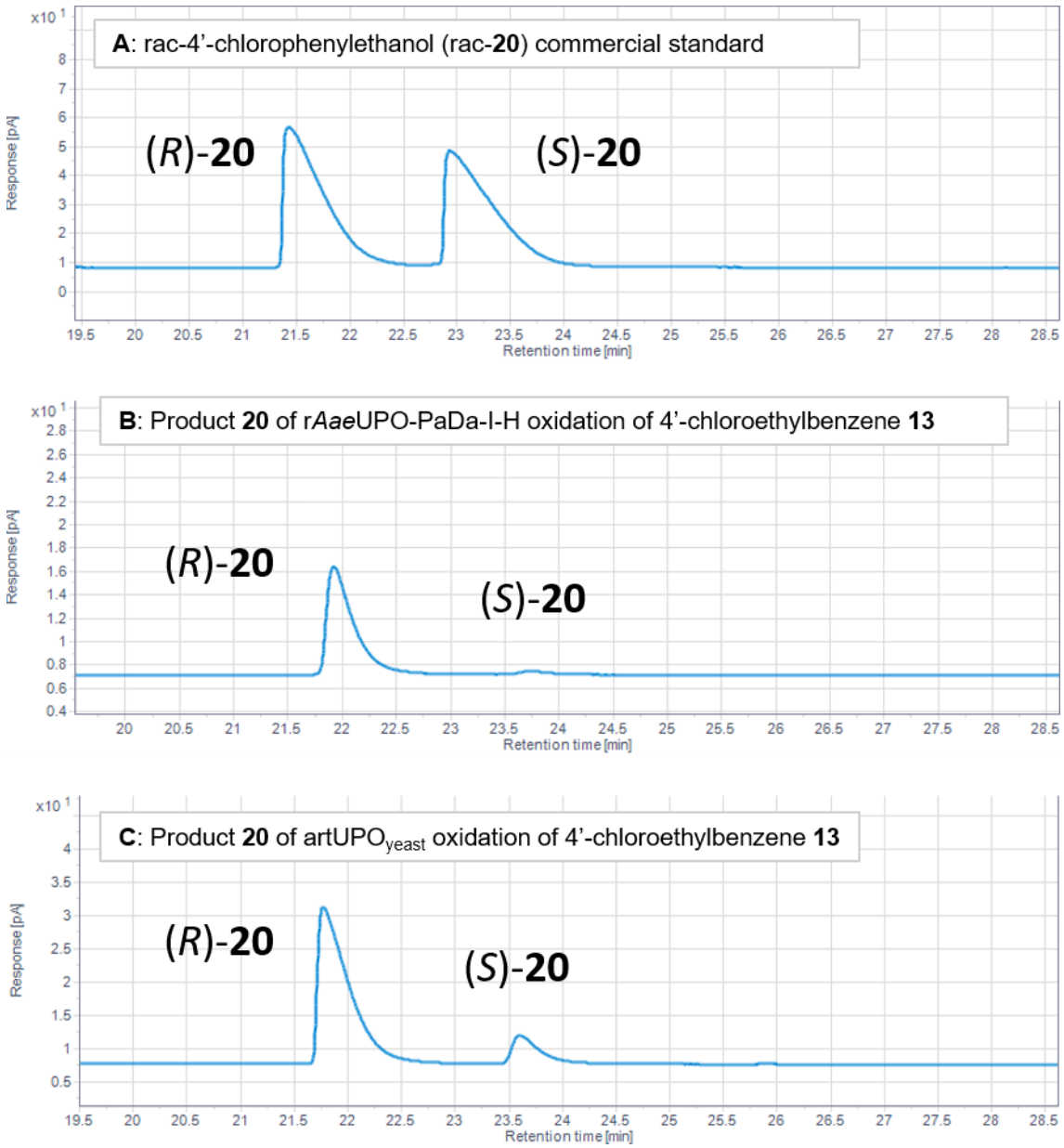

Figure S16. Chiral separation of enantiomers of **20**.

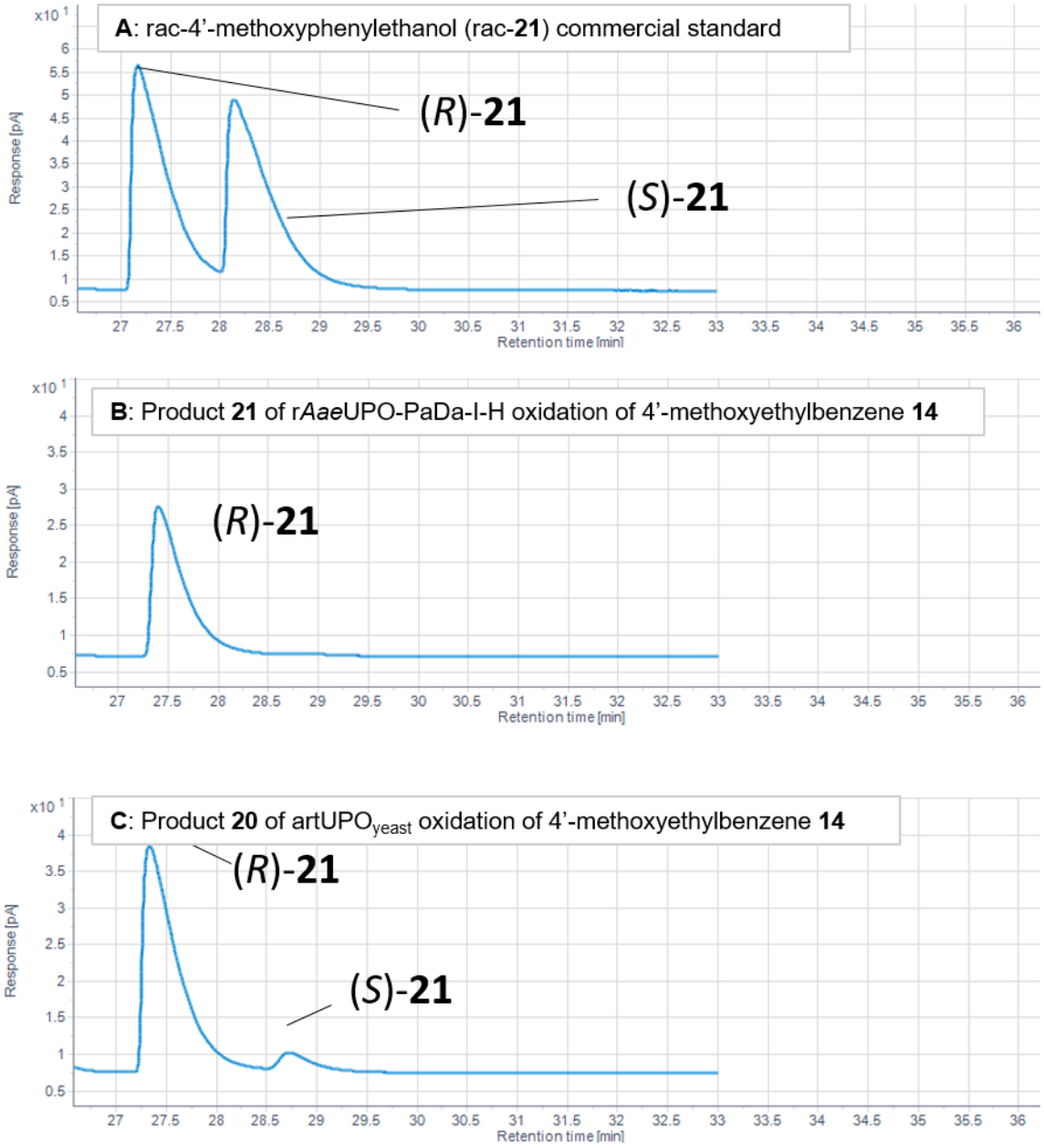

Figure S17. Chiral separation of enantiomers of **21**.

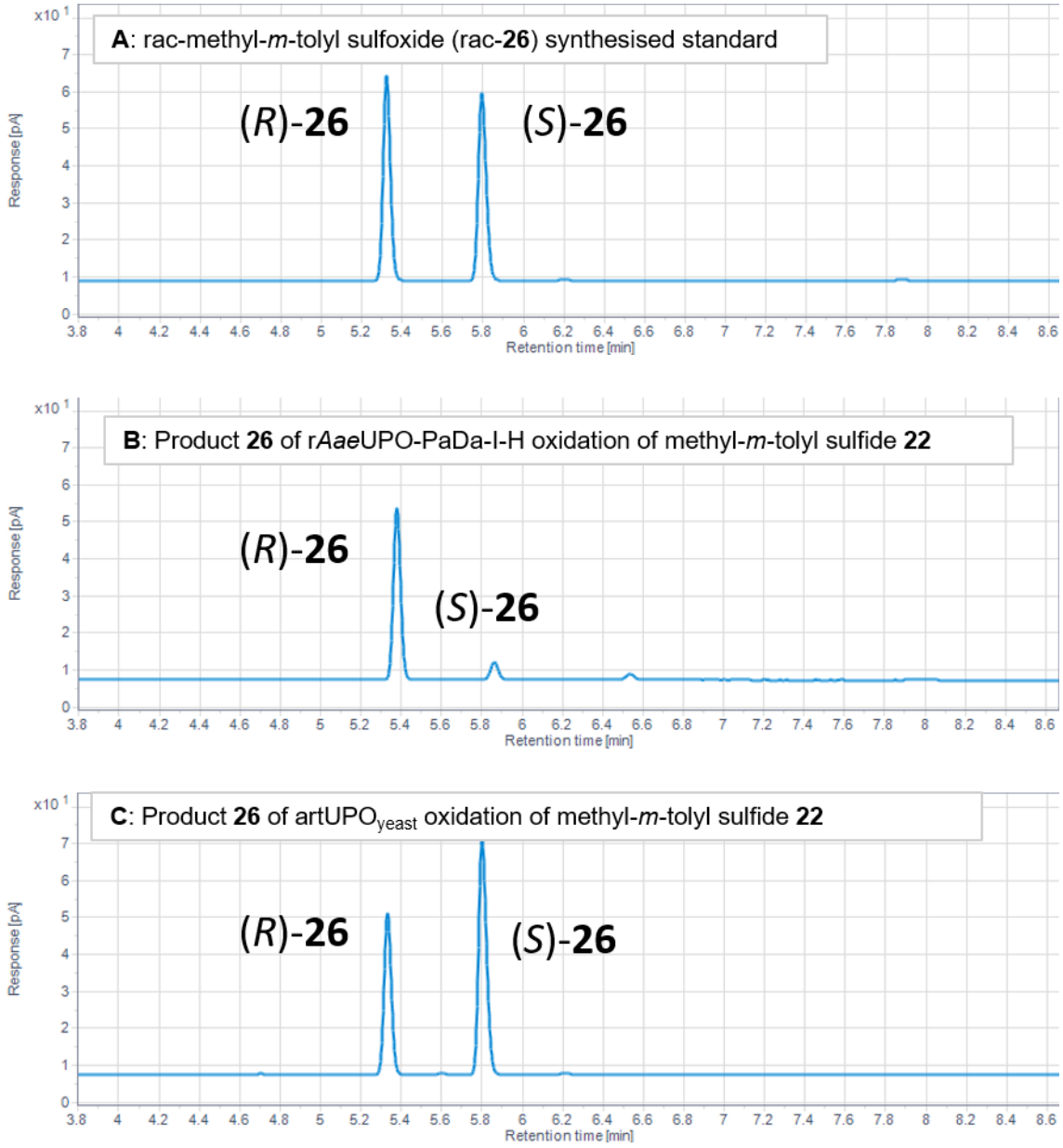

Figure S18. Chiral separation of enantiomers of **26**.

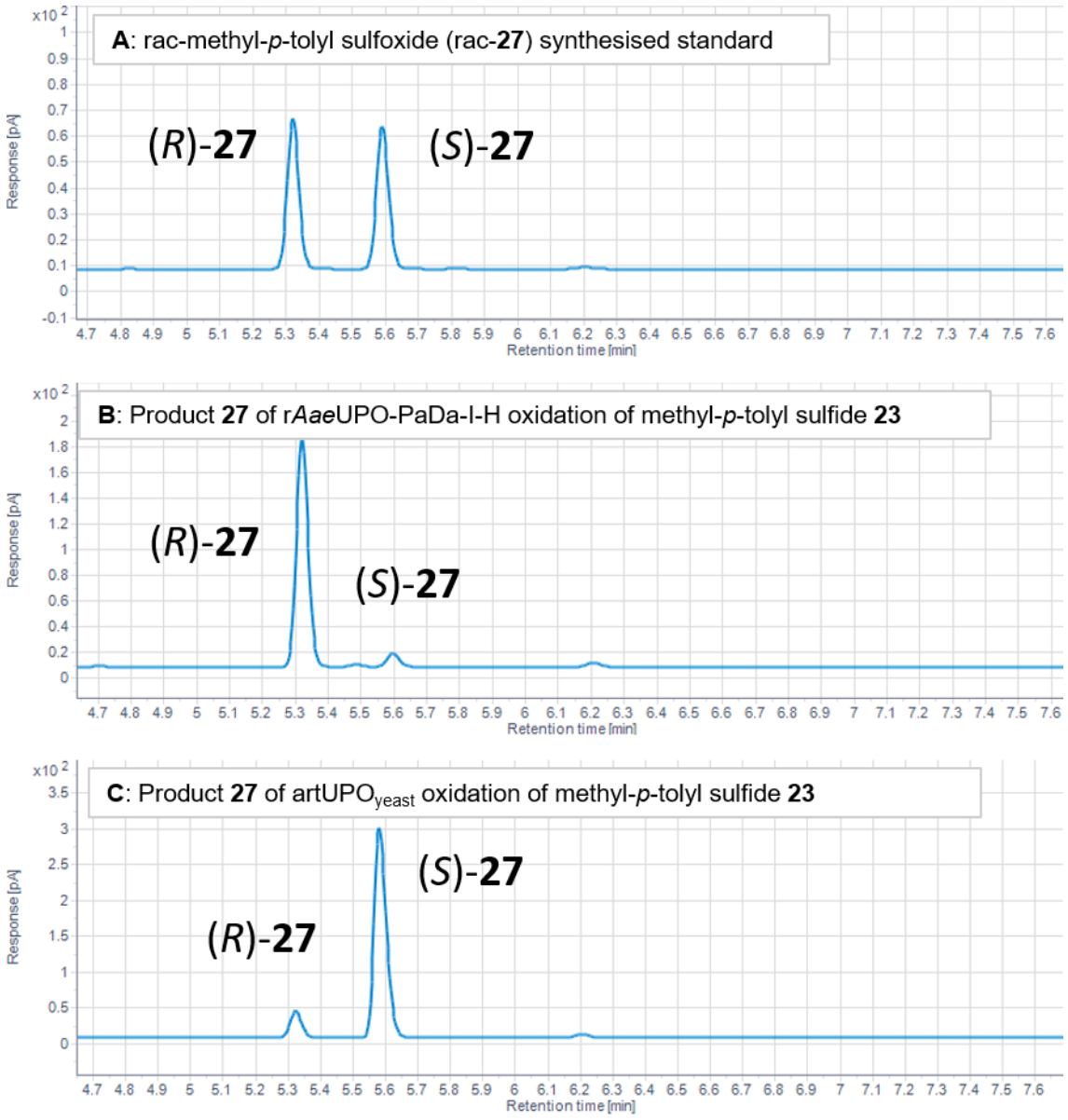

Figure S19. Chiral separation of enantiomers of **27**.

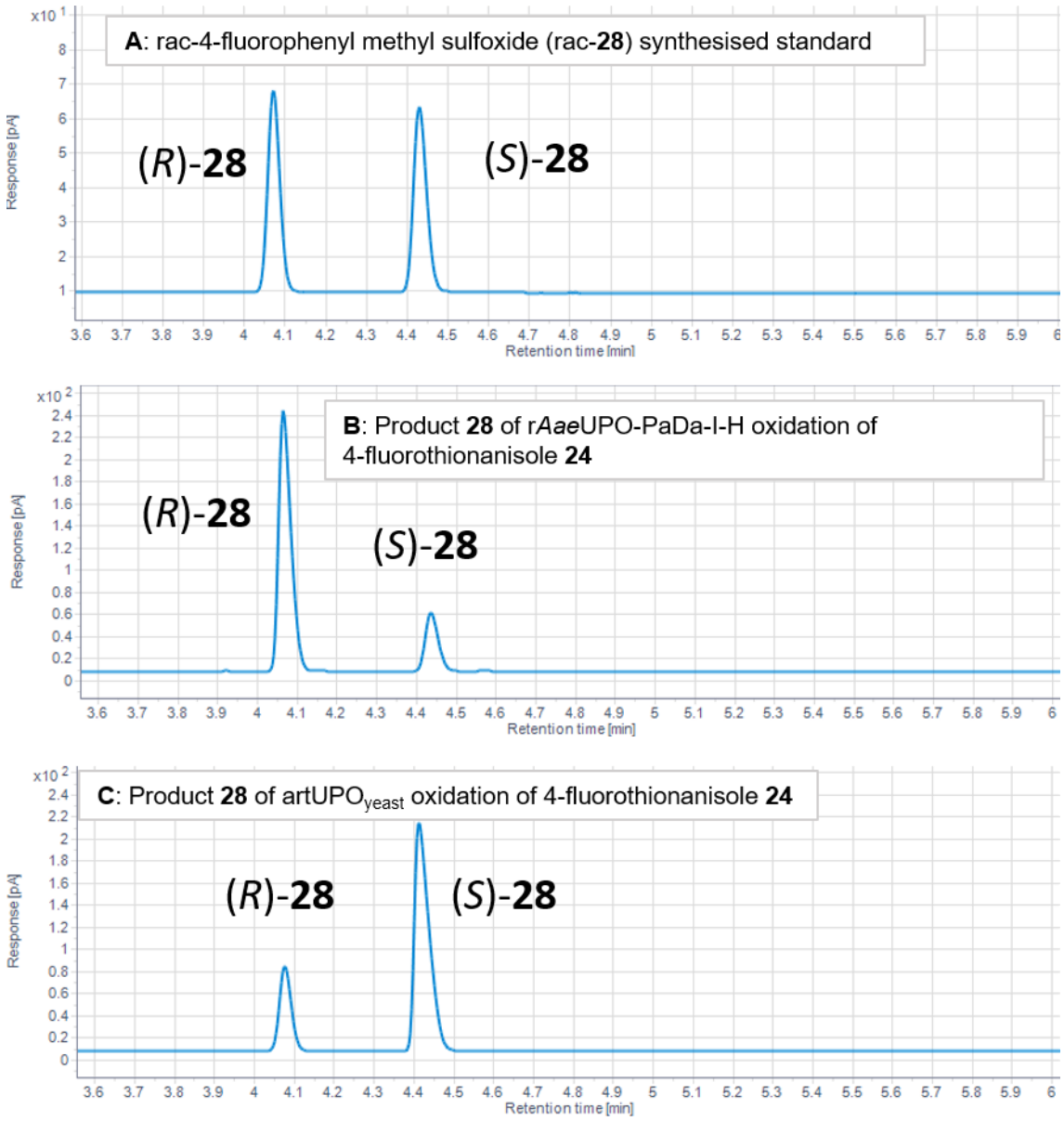

Figure S20. Chiral separation of enantiomers of **28**.

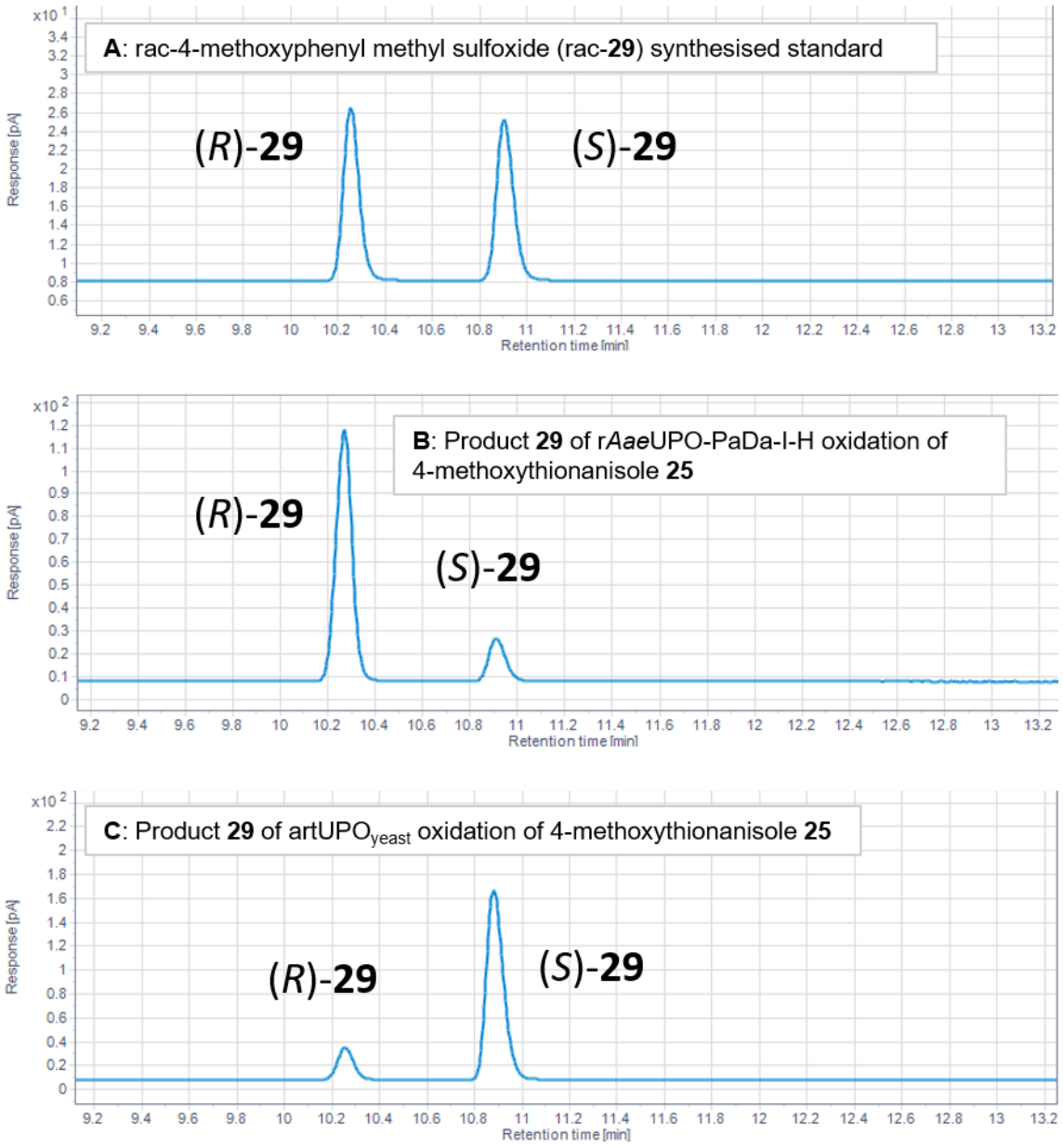

Figure S21. Chiral separation of enantiomers of **29**.

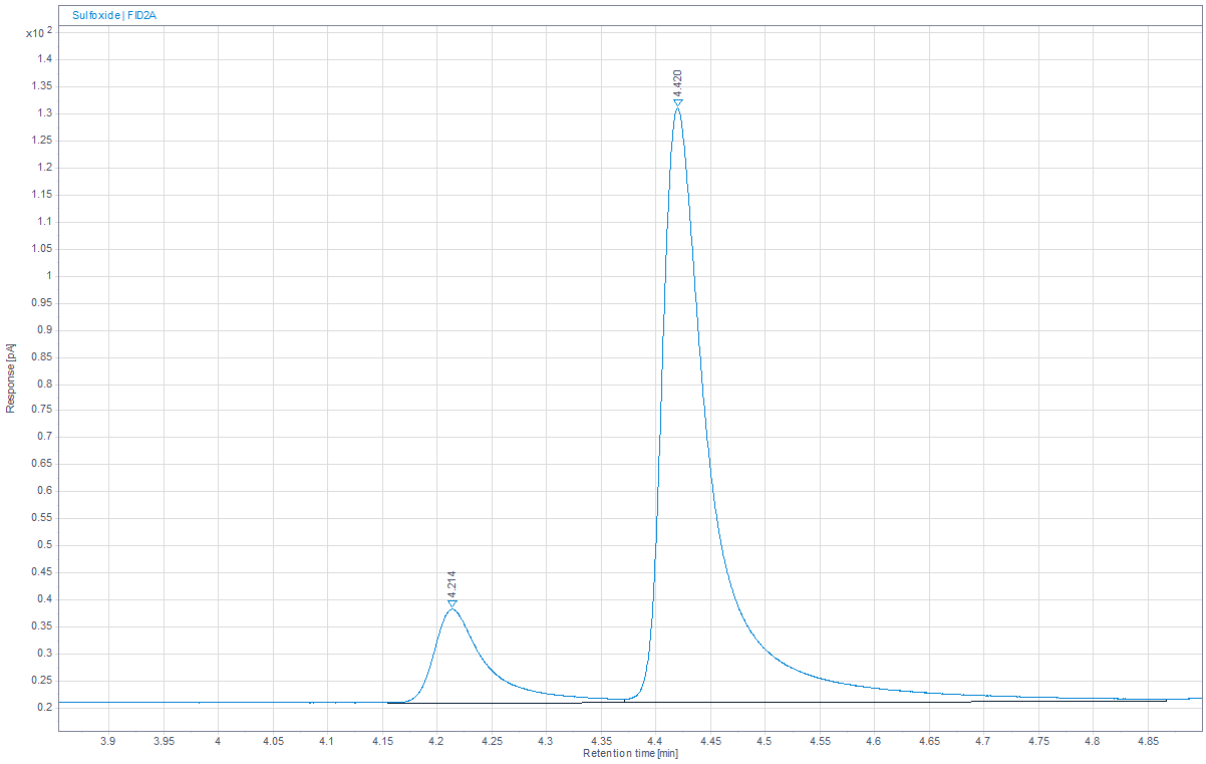

Figure S22. Chiral separation of enantiomers of **27** produced by artUPO$_{bact.}$