

Electronic Supporting Information for

Characterization of the Laccase-mediated Oligomerization of 4-Hydroxybenzoic Acid

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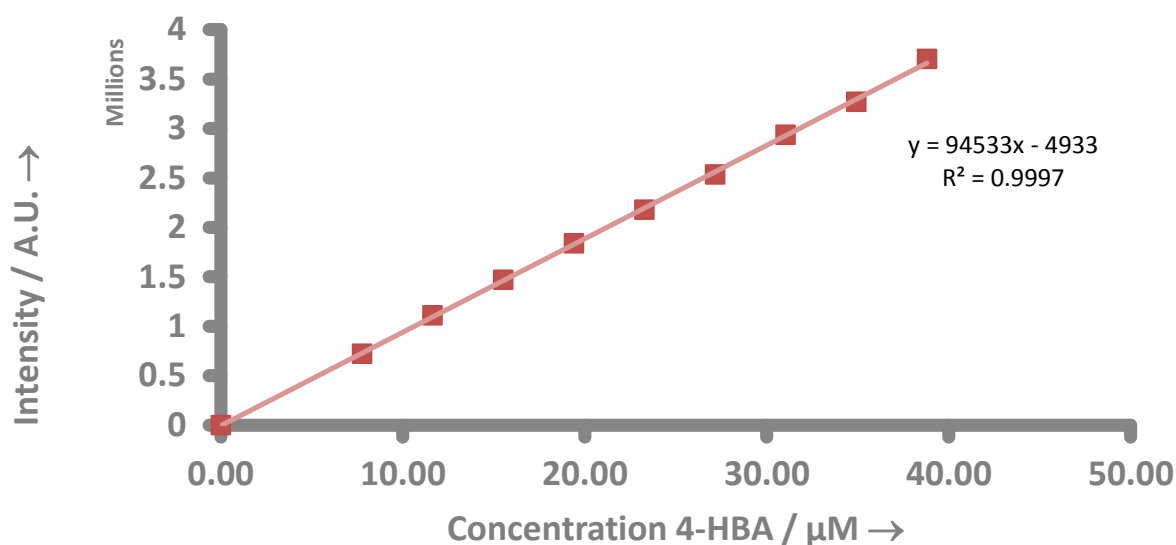
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Figure S1 - Calibration curve for determination of 4-HBA conversion.	4
Figure S2 - Calibration curve for determination of C3-C3' dimer 1 conversion.	5
Figure S3 - Calibration curve for determination of C3-O dimer 2 conversion.	6
Figure S4 - Calibration curve for determination of C1-C3' dimer 3 conversion.	7
Figure S5 - Calibration curve for determination of C1-O dimer 4 conversion.	7
Figure S6 - Extracted ion chromatogram for 137 ± 0.50 Da (4-HBA).	8
Figure S7 - Mass spectrum corresponding to peak at 3.7 min in SI figure S6.	8
Figure S8 - Extracted ion chromatogram for 273 ± 0.50 Da (dimers).	9
Figure S9 - Mass spectrum corresponding to peak at 4.5 min in SI figure S8.	9
Figure S10 - Mass spectrum corresponding to peak at 11.8 min in SI figure S8.	10
Figure S11 - Extracted ion chromatogram for 379 ± 0.50 Da (putative trimeric benzoquinone).	11
Figure S12 - Mass spectrum corresponding to peak at 13.3 min in SI figure S11.	11
Figure S13 - Extracted ion chromatogram for 545 ± 0.50 Da.	12
Figure S14 - Mass spectrum corresponding to peak at 12.0 min in SI figure S13.	12
Figure S15 - Extracted ion chromatogram for 229 ± 0.50 Da (dimer 3 and 4).	13
Figure S16 - Mass spectrum corresponding to peak at 7.7 min in SI figure S15.	13
Figure S17 - Co-injection dimer 1 and 2 with 4-HBA reaction.	14
Figure S18 - Co-injection dimer 3 with 4-HBA reaction.	14
Figure S19 - Co-injection dimer 4 with 4-HBA reaction.	15
Figure S20 - Mass spectrum for chemically synth. dimer 1 at retention time 4.5 min (LC1).	16
Figure S21 - Mass spectrum for chemically synth. dimer 2 at retention time 11.8 min (LC1).	16
Figure S22 - Mass spectrum for chemically synthesized dimer 3 at retention time 7.7 min (LC3).	17
Figure S23 - Yield of dimer 3 in the laccase-mediated oligomerization of 4-HBA over time.	17
Figure S24 - Conversion of dimer 3 by laccase over time.	18
Figure S25 - Conversion of dimer 4 by laccase over time.	19
Figure S26 - ^1H NMR spectrum of methylated precursor of dimer 1 (solvent: CDCl_3).	20
Figure S27 - ^1H NMR spectrum of methylated precursor of dimer 2 (solvent: CDCl_3).	20
Figure S28 - ^1H NMR spectrum of chemically synthesized dimer 1 (solvent: acetone- d_6).	21
Figure S29 - ^1H NMR spectrum of enzymatically generated dimer 1 (solvent: acetone- d_6).	21
Figure S30 - ^1H NMR spectrum of chemically synthesized dimer 2 (solvent: CD_3OD).	22
Figure S31 - ^1H NMR spectrum of enzymatically generated dimer 2 (solvent: CD_3OD).	22
Figure S32 - ^{13}C NMR spectrum of methylated precursor of dimer 1 (solvent: CDCl_3).	23
Figure S33 - ^{13}C NMR spectrum of methylated precursor of dimer 2 (solvent: CDCl_3).	23
Figure S34 - ^{13}C NMR spectrum of chemically synthesized dimer 1 (solvent: CD_3OD).	24

Figure S35 - ^{13}C NMR spectrum of chemically synthesized dimer 2 (solvent: acetone- d_6).	24
Figure S36 - ^1H NMR spectrum of chemically synthesized dimer 3 (solvent: acetone- d_6).	25
Figure S37 - ^{13}C NMR spectrum of chemically synthesized dimer 3 (solvent: acetone- d_6).	25
Figure S38 - Optimized geometries for 4-hydroxybenzoic acid, dimer 1 and dimer 2.	26
Table S1 - Calculated relative energies for the formation of the radical cation.	26
Table S2 - Comparison of ^1H NMR data for dimers 1 and 2.	27

Figure S1 - Calibration curve for determination of 4-HBA conversion.

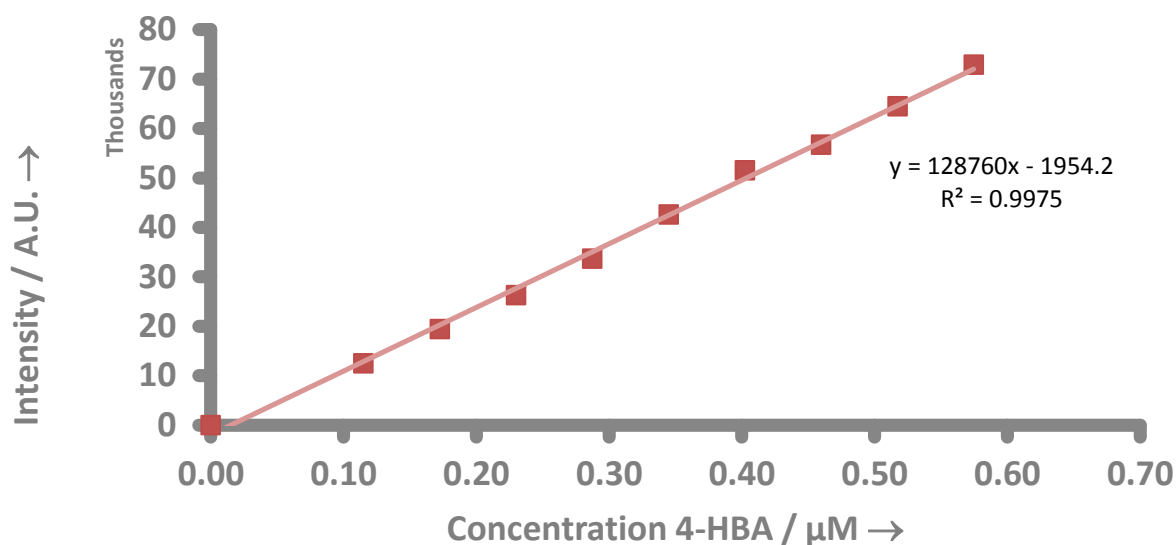


Measured peak areas (UV chromatogram, 254 nm) for the peak corresponding to 4-hydroxybenzoic acid at several known concentrations (0.00 μM , 7.76 μM , 11.64 μM , 15.52 μM , 19.40 μM , 23.28 μM , 27.16 μM , 31.05 μM , 34.93 μM , 38.81 μM). The calibration curve is fitted along these points using the least squares method according to the formula $y = ax + b$.

a (slope) and b (intercept at $x = 0$) are 94533 and -4933 respectively.

The measured intensity y for 4-HBA after the 24 h enzymatic reaction (after 1250 \times dilution): 153881. This results in $x_{\text{after_reaction}} = 16.24 \mu\text{M}$ from $x_{\text{before_reaction}}$ (diluted sample) = concentration 4-HBA at time 0 = 21.89 μM , which means 26% 4-HBA has been converted.

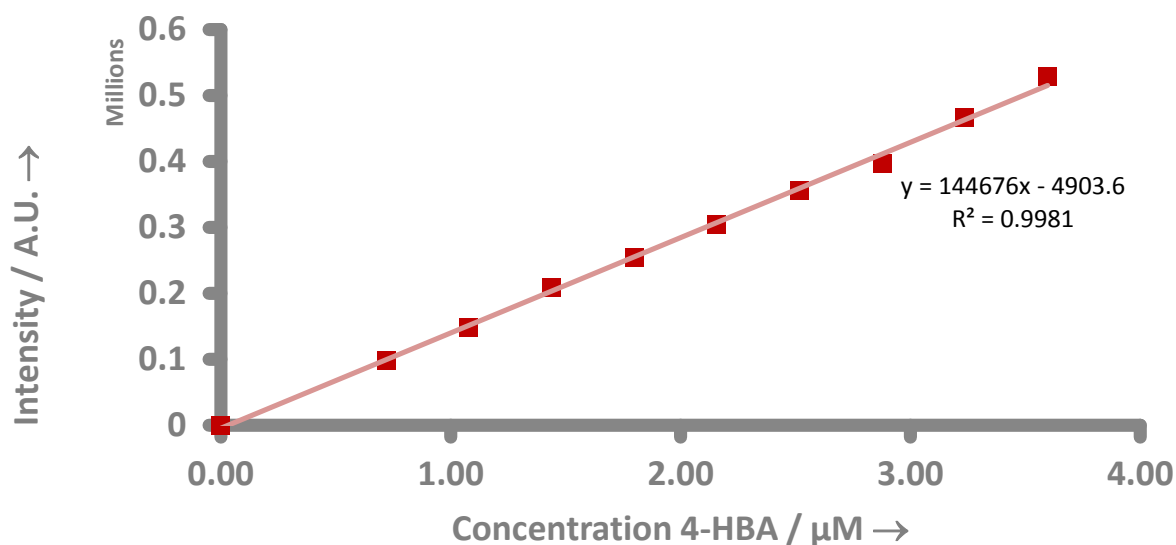
Figure S2 - Calibration curve for determination of C3-C3' dimer 1 conversion.



Measured peak areas (UV chromatogram, 254 nm) for the peak corresponding to C3-C3' dimer **1** at several known concentrations (0.00 μM , 0.11 μM , 0.17 μM , 0.23 μM , 0.29 μM , 0.34 μM , 0.40 μM , 0.46 μM , 0.52 μM , 0.57 μM). The calibration curve is fitted along these points using the least squares method according to the formula $y = ax + b$. a (slope) and b (intercept at $x = 0$) are 128760 and -1954.2 respectively.

The measured intensity y for C3-C3' dimer **1** after the 24 h enzymatic reaction (after 1250 \times dilution): 26850. This results in $x_{\text{after_reaction}} = 0.22 \mu\text{M}$ from $x_{\text{before_reaction}}$ (diluted sample) = concentration 4-HBA at time 0 = 21.12 μM , which means 2% 4-HBA has been converted to C3-C3' dimer **1** (2 units of 4-HBA convert to 1 unit of dimer).

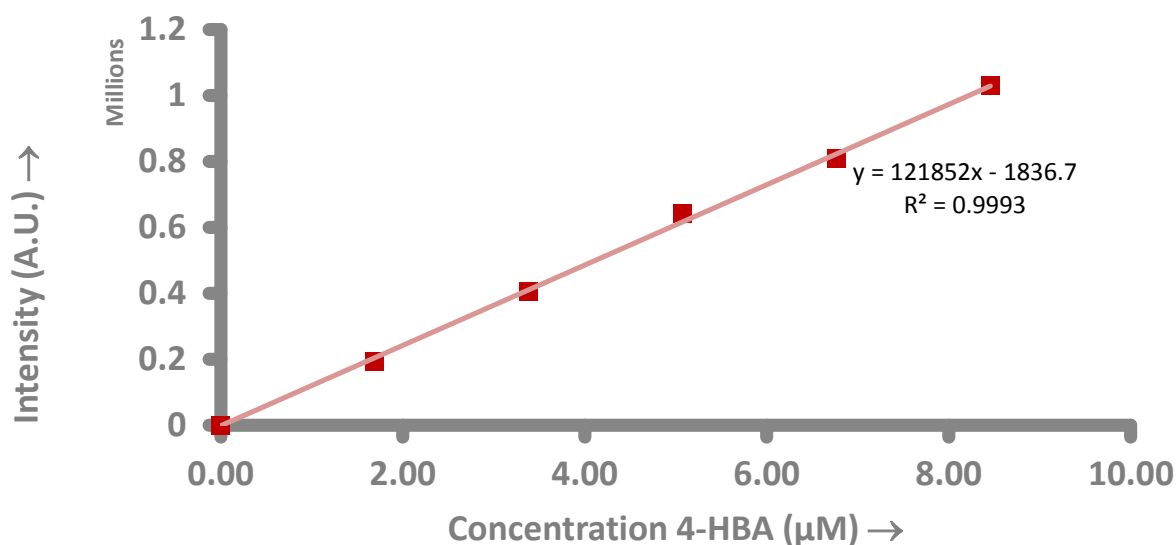
Figure S3 - Calibration curve for determination of C3-O dimer 2 conversion.



Measured peak areas (UV chromatogram, 254 nm) for the peak corresponding to C3-O dimer **2** at several known concentrations (0.00 μM , 0.72 μM , 1.08 μM , 1.44 μM , 1.80 μM , 2.16 μM , 2.52 μM , 2.88 μM , 3.24 μM , 3.60 μM). The calibration curve is fitted along these points using the least squares method according to the formula $y = ax + b$. a (slope) and b (intercept at $x = 0$) are 144676 and -4903.6 respectively.

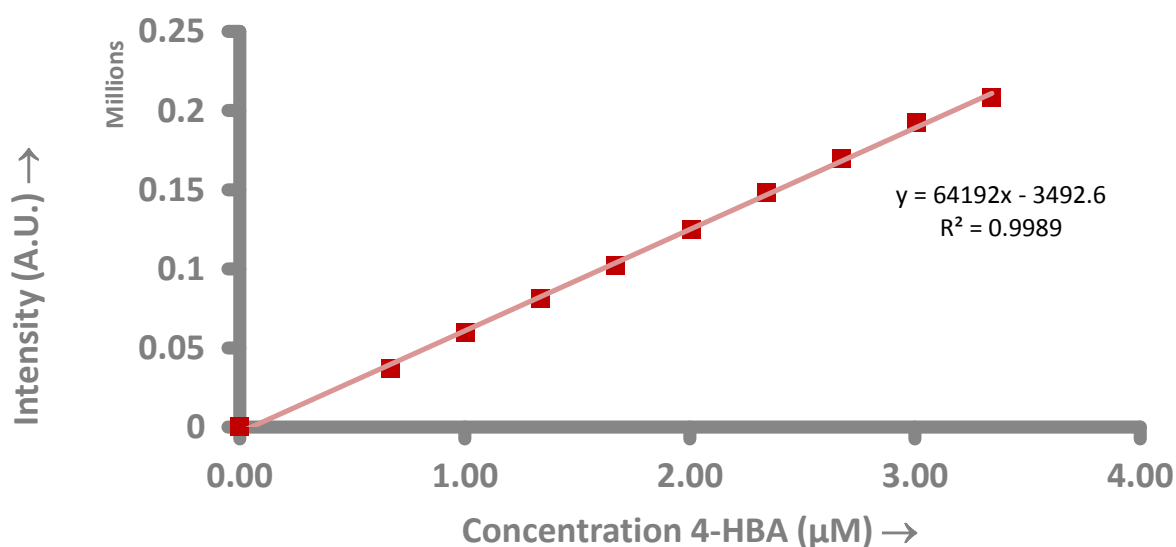
The measured intensity y for C3-O dimer **2** after the 24 h enzymatic reaction (after 10 \times dilution): 444224. This results in $x_{\text{after_reaction}} = 3.07 \mu\text{M}$ from $x_{\text{before_reaction}}$ (diluted sample) = concentration 4-HBA at time 0 = 2640 μM , which means 0.2% 4-HBA has been converted to C3-O dimer **2** (2 units of 4-HBA convert to 1 unit of dimer).

Figure S4 - Calibration curve for determination of C1-C3' dimer 3 conversion.

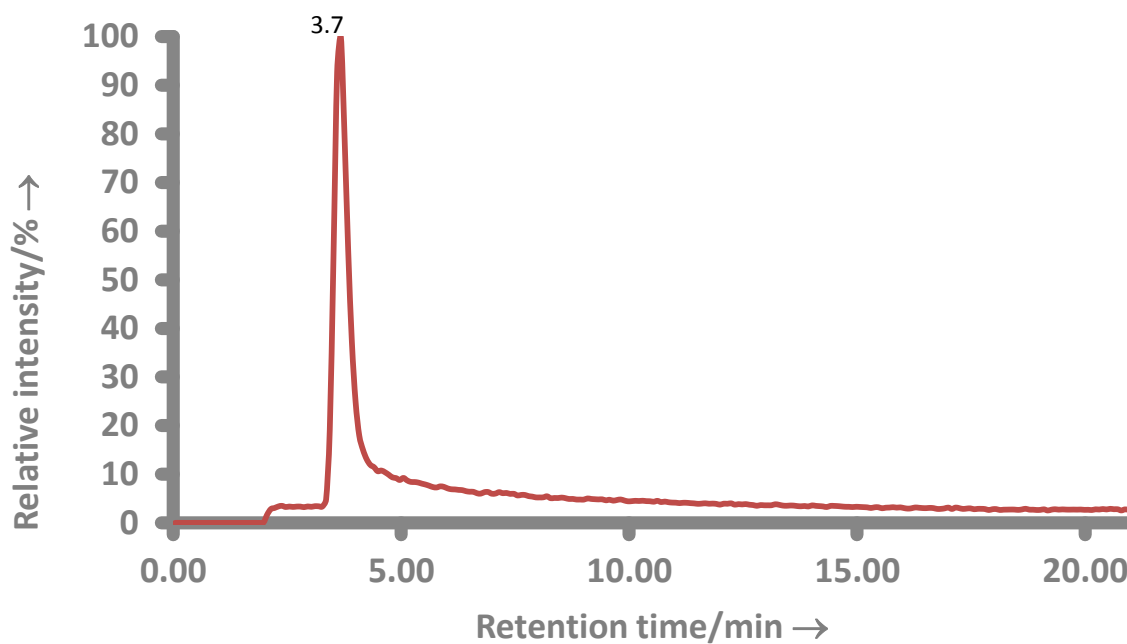


Measured peak areas (UV chromatogram, 254 nm) for the peak corresponding to C1-C3' dimer **3** at several known concentrations (0.00 µM, 1.69 µM, 3.38 µM, 5.07 µM, 6.77 µM, 8.46 µM). The calibration curve is fitted along these points using the least squares method according to the formula $y = ax + b$. a (slope) and b (intercept at $x = 0$) are 121852 and -1836.7 respectively.

Figure S5 - Calibration curve for determination of C1-O dimer 4 conversion.



Measured peak areas (UV chromatogram, 254 nm) for the peak corresponding to C1-O dimer **4** at several known concentrations (0.00 µM, 0.67 µM, 1.00 µM, 1.34 µM, 1.67 µM, 2.00 µM, 2.34 µM, 2.67 µM, 3.01 µM, 3.34 µM). The calibration curve is fitted along these points using the least squares method according to the formula $y = ax + b$. a (slope) and b (intercept at $x = 0$) are 64192 and -3492.6 respectively.

Figure S6 - Extracted ion chromatogram for 137 ± 0.50 Da (4-HBA).

Extracted ion chromatogram for 137 ± 0.50 Da (4-HBA) from LC-MS analysis of 4-HBA oligomerization (LC-separation: LC1, line smoothing applied for clarity).

Figure S7 - Mass spectrum corresponding to peak at 3.7 min in SI figure S6.

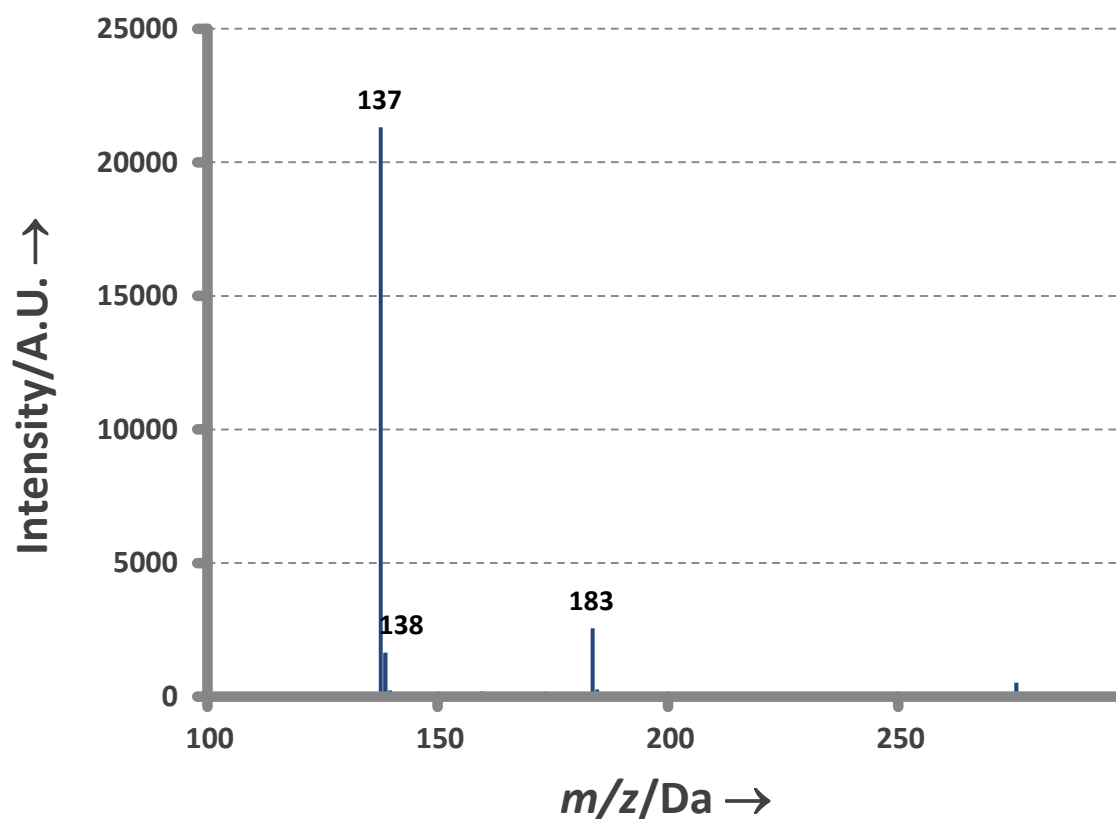
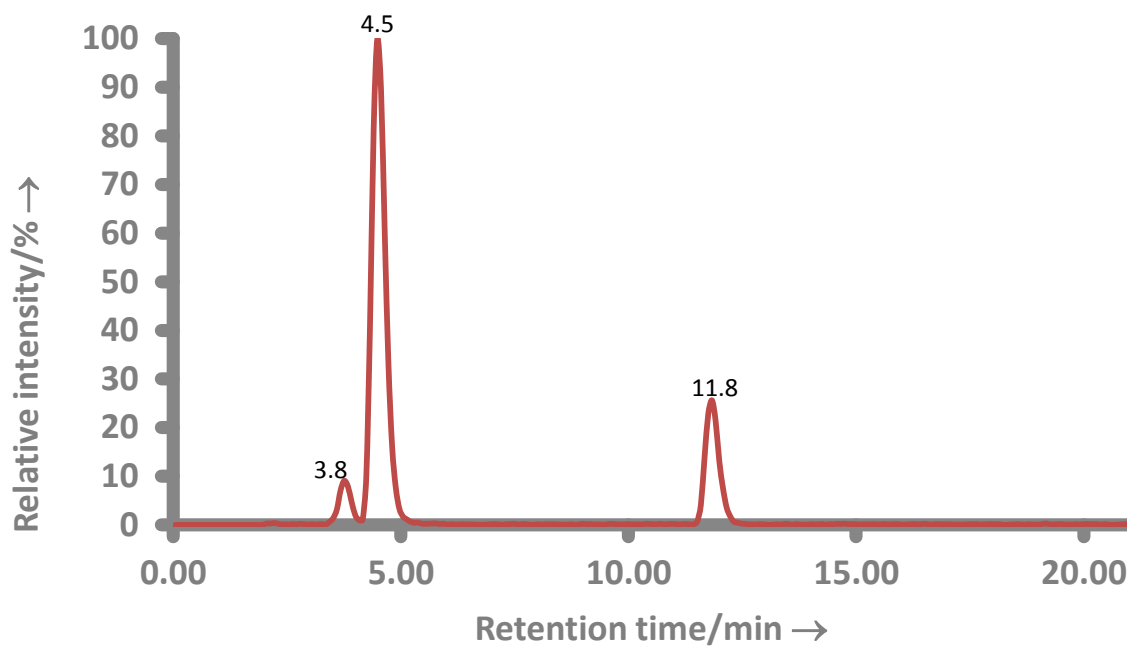


Figure S8 - Extracted ion chromatogram for 273 ± 0.50 Da (dimers).

Extracted ion chromatogram for 273 ± 0.50 Da (dimers) from LC-MS analysis of 4-HBA oligomerization (LC-separation: LC1, line smoothing applied for clarity).

Figure S9 - Mass spectrum corresponding to peak at 4.5 min in SI figure S8.

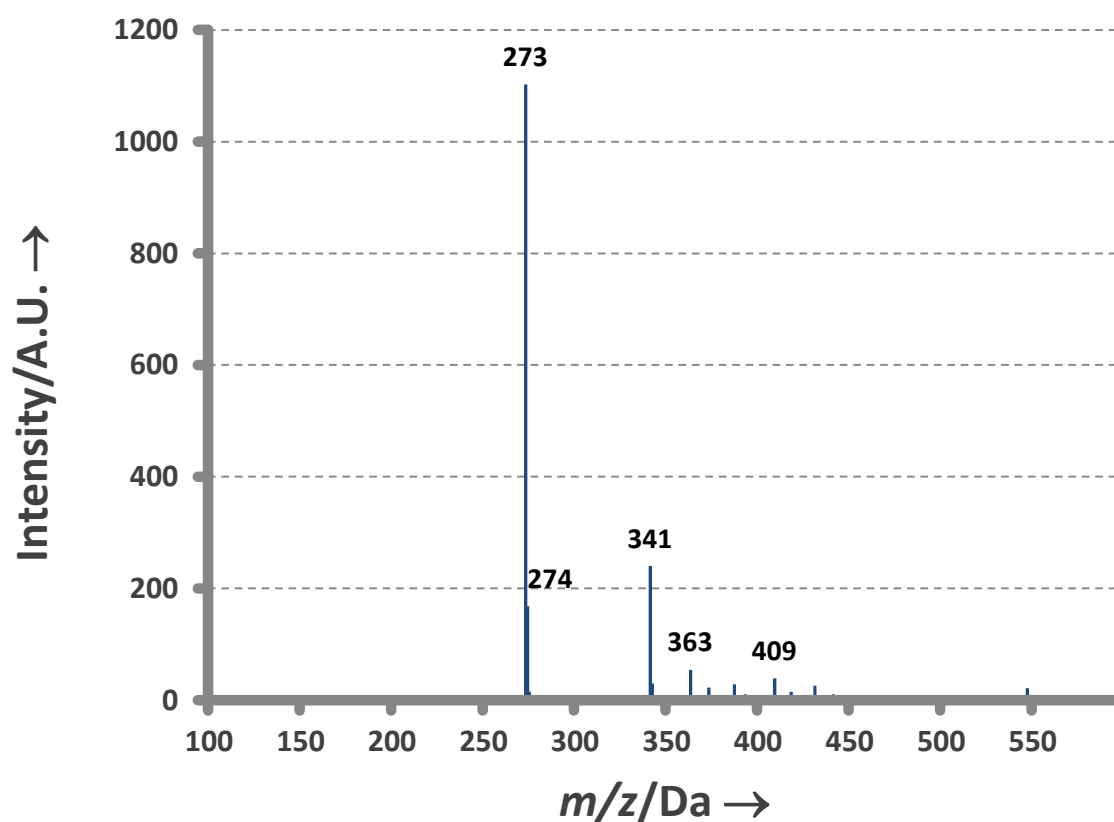


Figure S10 - Mass spectrum corresponding to peak at 11.8 min in SI figure S8.

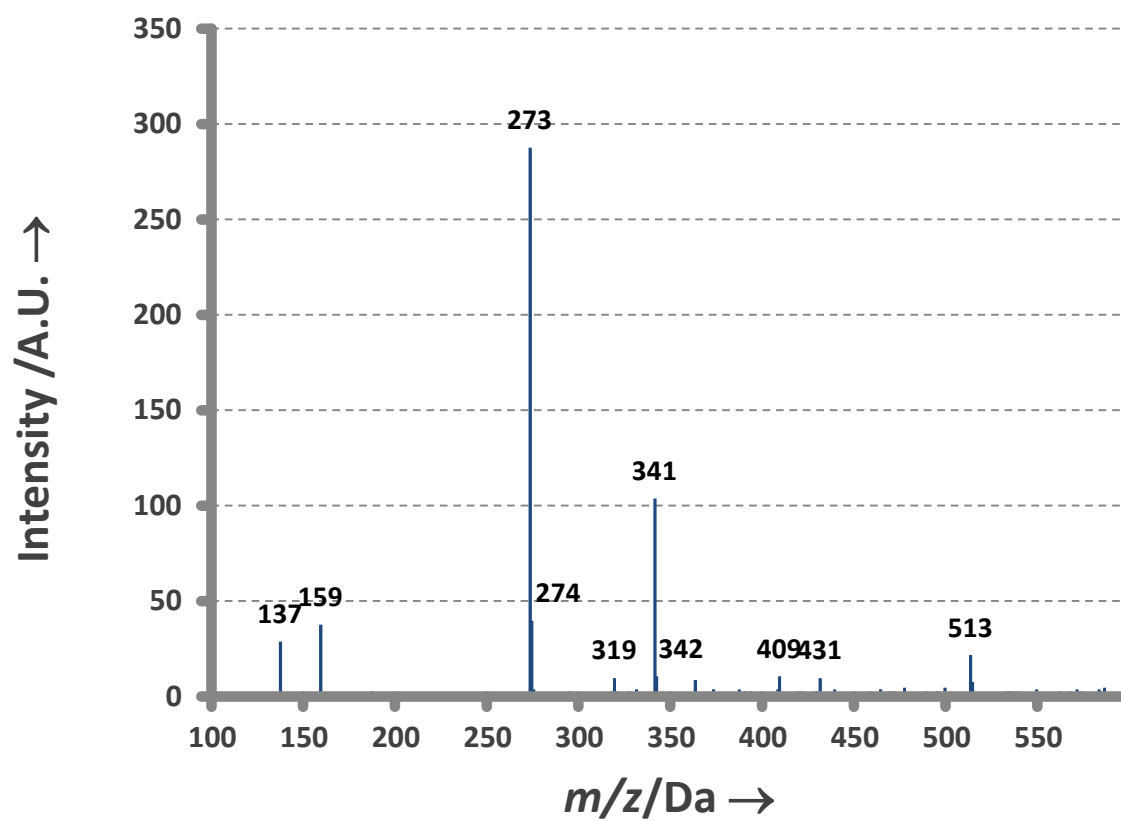
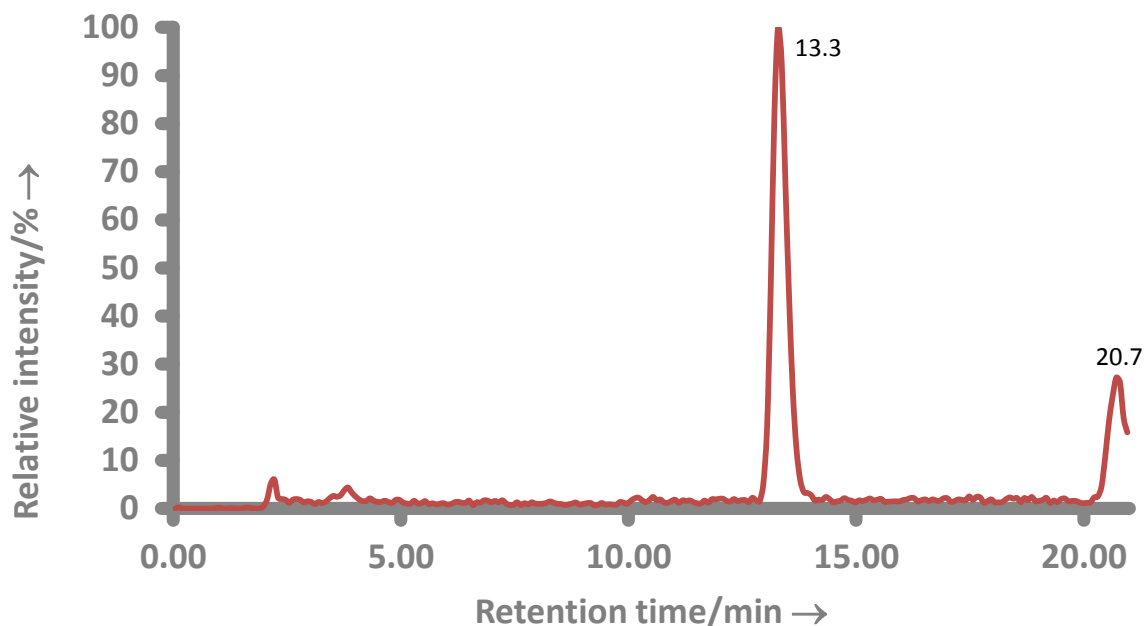


Figure S11 - Extracted ion chromatogram for 379 ± 0.50 Da (putative trimeric benzoquinone).



Extracted ion chromatogram for 379 ± 0.50 Da (putative trimeric benzoquinone) from LC-MS analysis of 4-HBA oligomerization (LC-separation: LC1, line smoothing applied for clarity).

Figure S12 - Mass spectrum corresponding to peak at 13.3 min in SI figure S11.

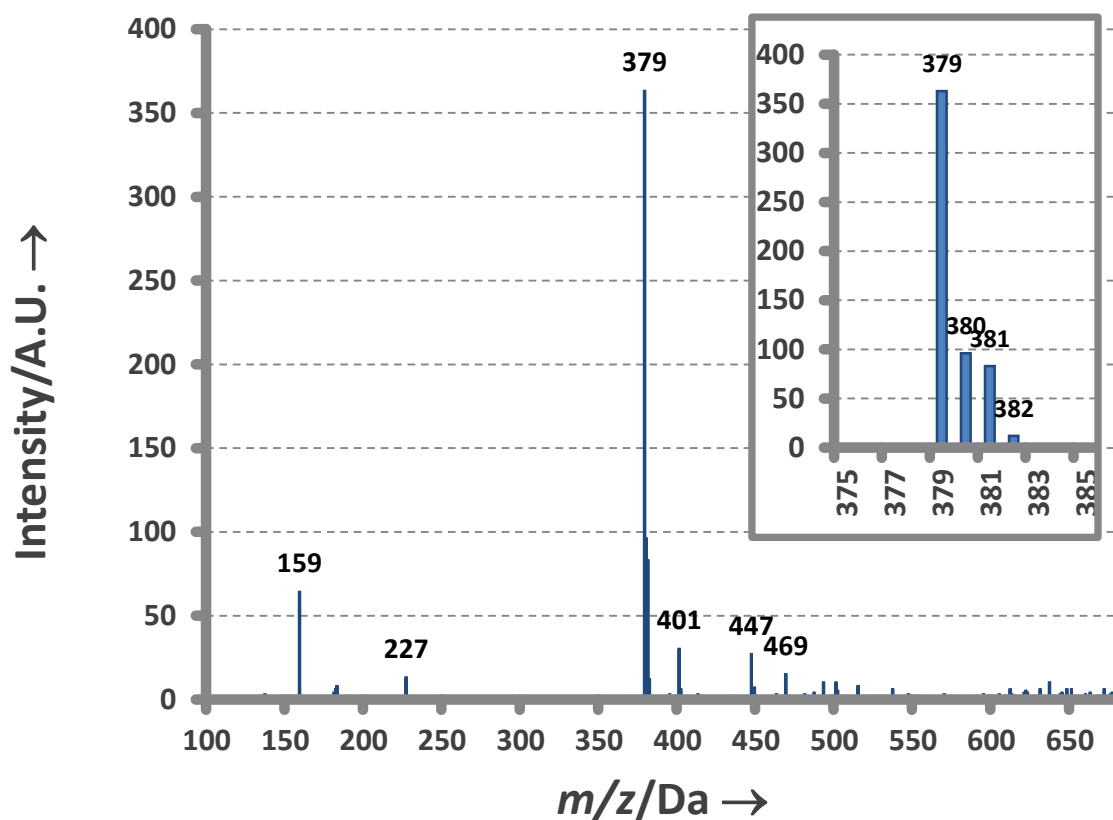
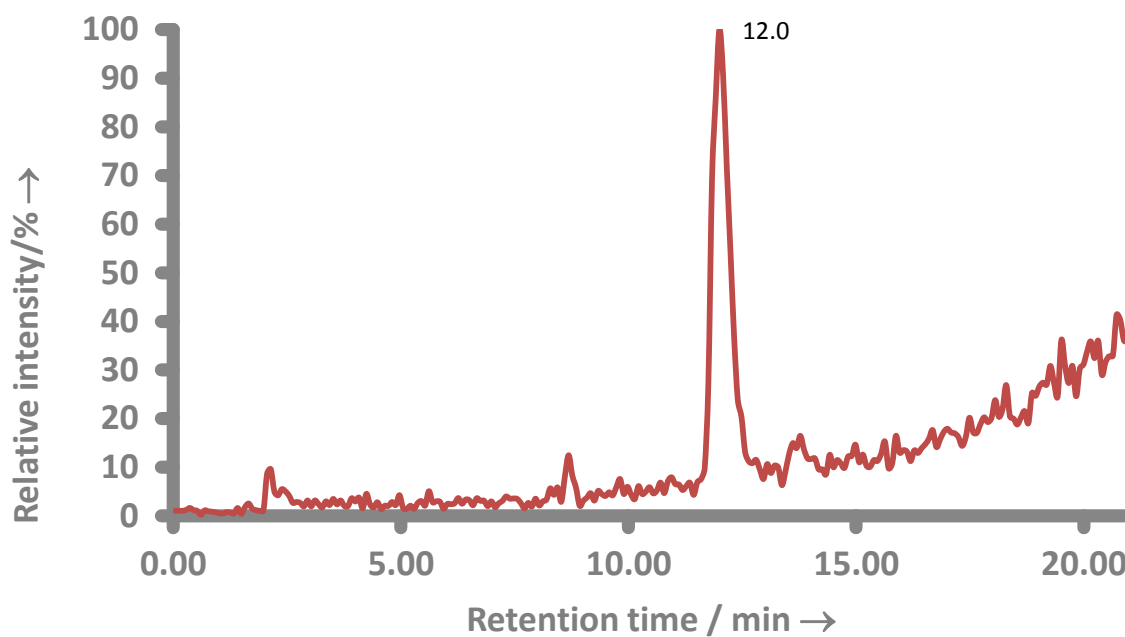


Figure S13 - Extracted ion chromatogram for 545 ± 0.50 Da.

Extracted ion chromatogram for 545 ± 0.50 Da from LC-MS analysis of laccase mediated conversion of 4-HBA and dimer **2** (LC-separation: LC2, line smoothing applied for clarity).

Figure S14 - Mass spectrum corresponding to peak at 12.0 min in SI figure S13.

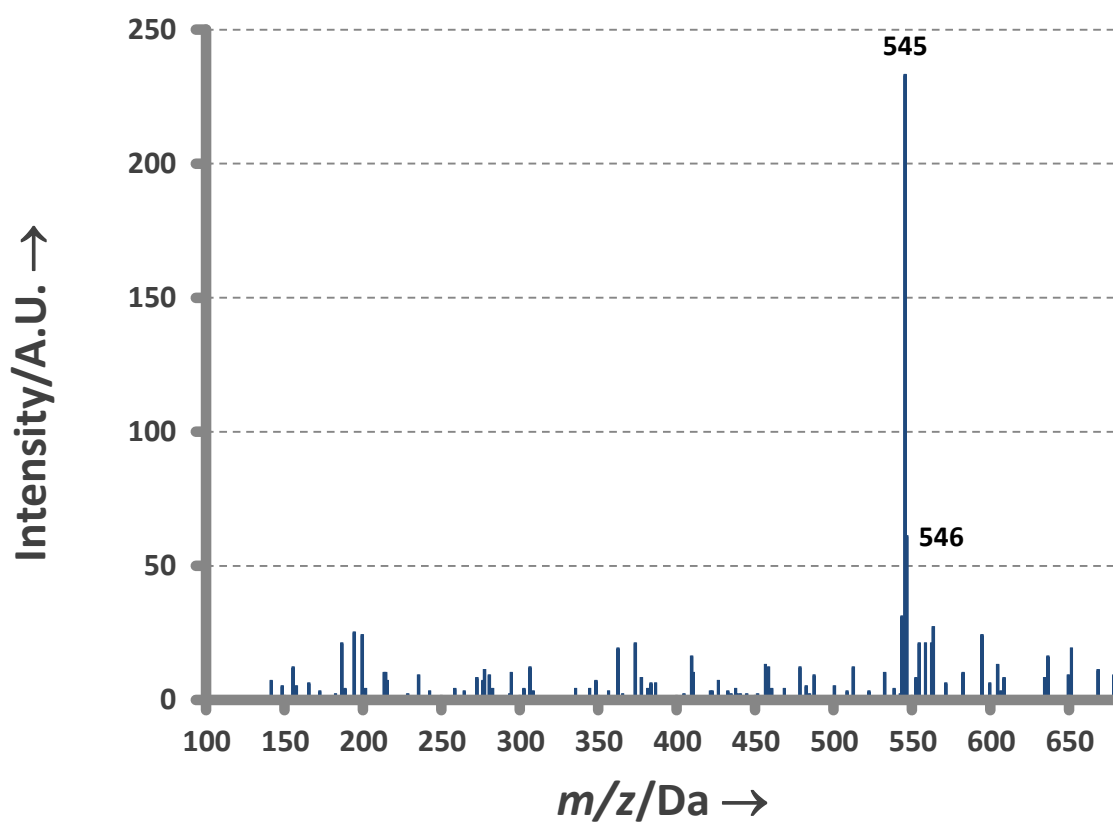
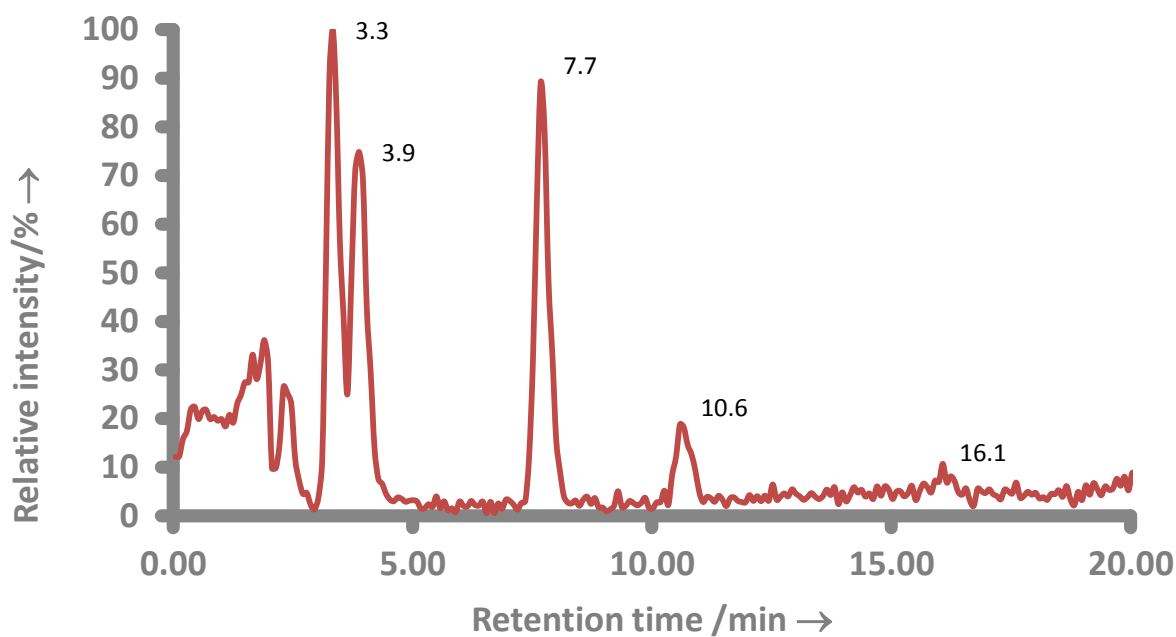


Figure S15 - Extracted ion chromatogram for 229 ± 0.50 Da (dimer **3** and **4**).

Extracted ion chromatogram for 229 ± 0.50 Da (dimer **3** and **4**) from LC-MS analysis of laccase mediated conversion of 4-HBA (LC-separation: LC3, line smoothing applied for clarity).

Figure S16 - Mass spectrum corresponding to peak at 7.7 min in SI figure S15.

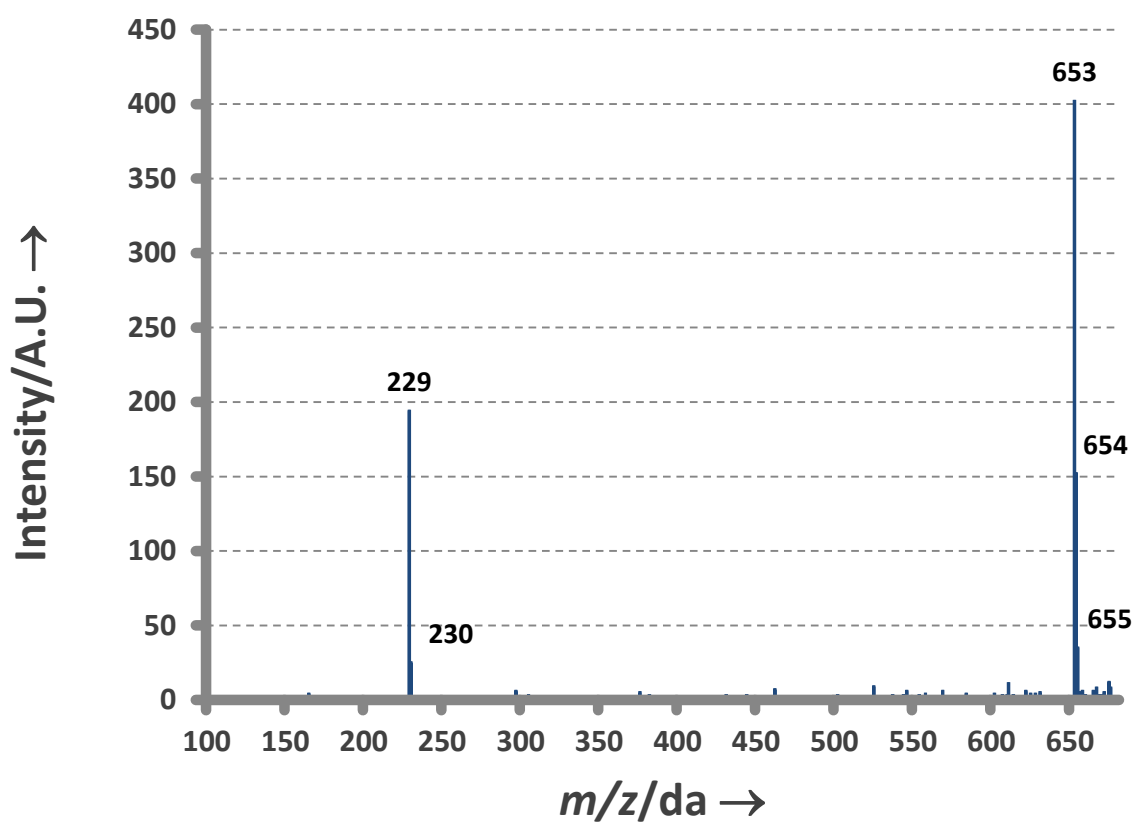
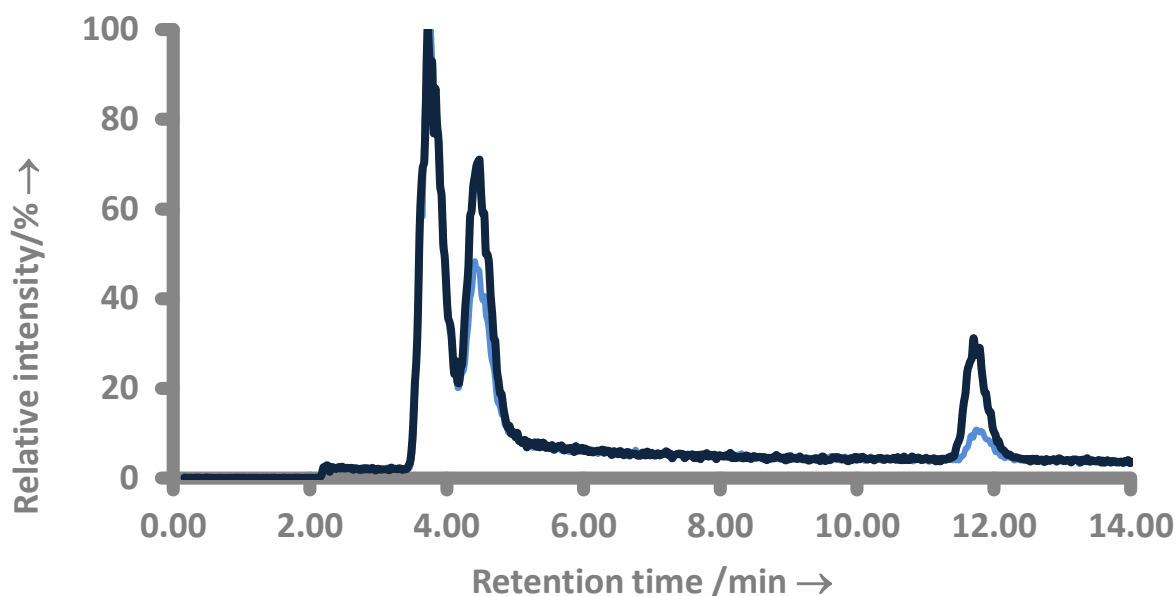
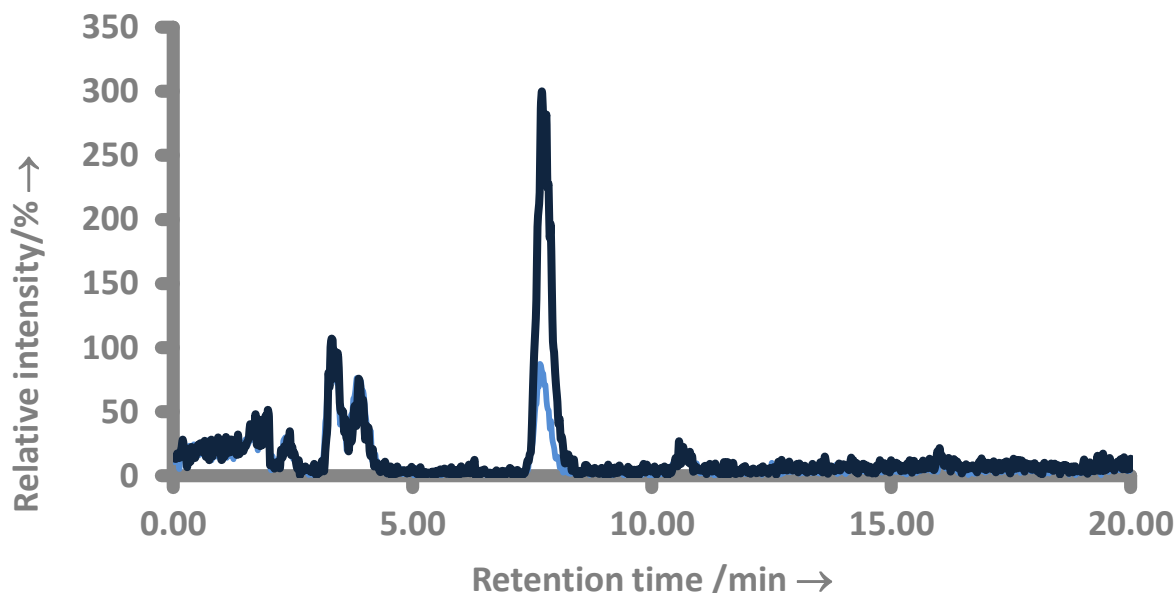


Figure S17 - Co-injection dimer 1 and 2 with 4-HBA reaction.

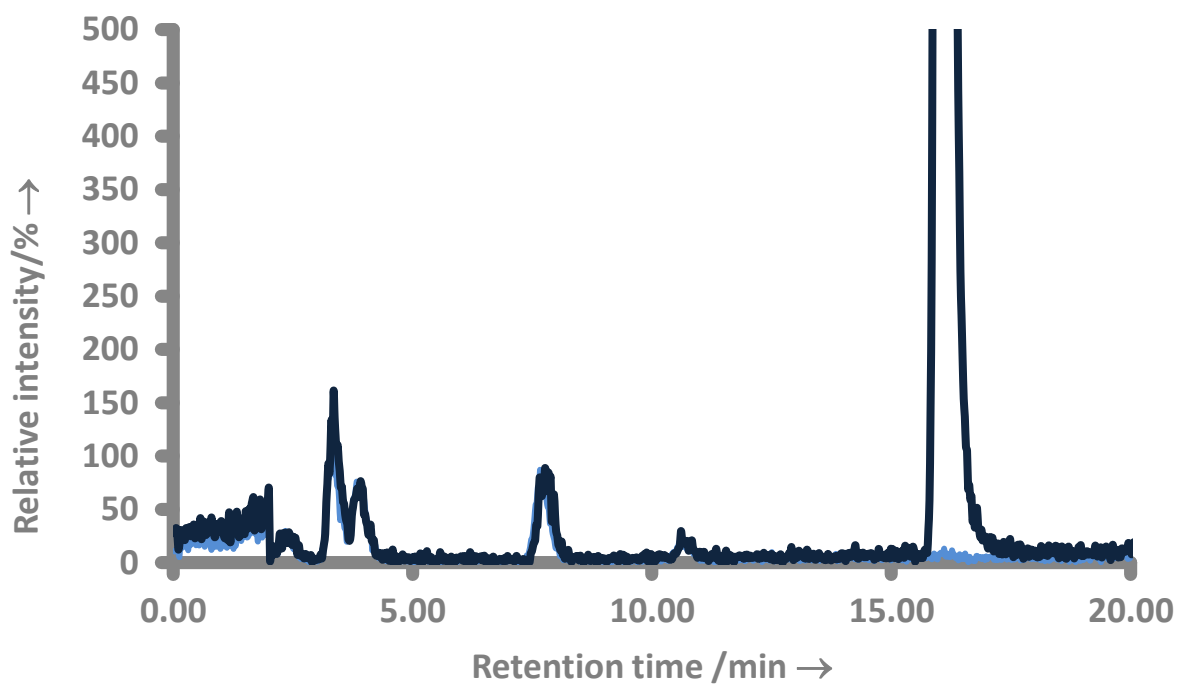


Comparison of extracted ion chromatograms ($m/z 137 \pm 0.50$ Da + $m/z 273 \pm 0.50$ Da) of the reaction mixture from laccase-mediated oligomerization of 4-hydroxybenzoic acid at 24 h (light blue line) and the same reaction mixture spiked with chemically synthesized dimer **1** and dimer **2** (dark blue line). Separation was achieved through LC-method: LC1.

Figure S18 - Co-injection dimer 3 with 4-HBA reaction.



Comparison of extracted ion chromatograms ($m/z 229 \pm 0.50$ Da) of the reaction mixture from laccase-mediated oligomerization of 4-hydroxybenzoic acid at 24 h (light blue line) and the same reaction mixture spiked with chemically synthesized dimer **3** (dark blue line). Separation was achieved through LC-method: LC3.

Figure S19 - Co-injection dimer 4 with 4-HBA reaction.

Comparison of extracted ion chromatograms ($m/z\ 229 \pm 0.50\ \text{Da}$) of the reaction mixture from laccase-mediated oligomerization of 4-hydroxybenzoic acid at 24 h (light blue line) and the same reaction mixture spiked with chemically synthesized dimer **4** (dark blue line). Separation was achieved through LC-method: LC3.

Figure S20 - Mass spectrum for chemically synth. dimer 1 at retention time 4.5 min (LC1).

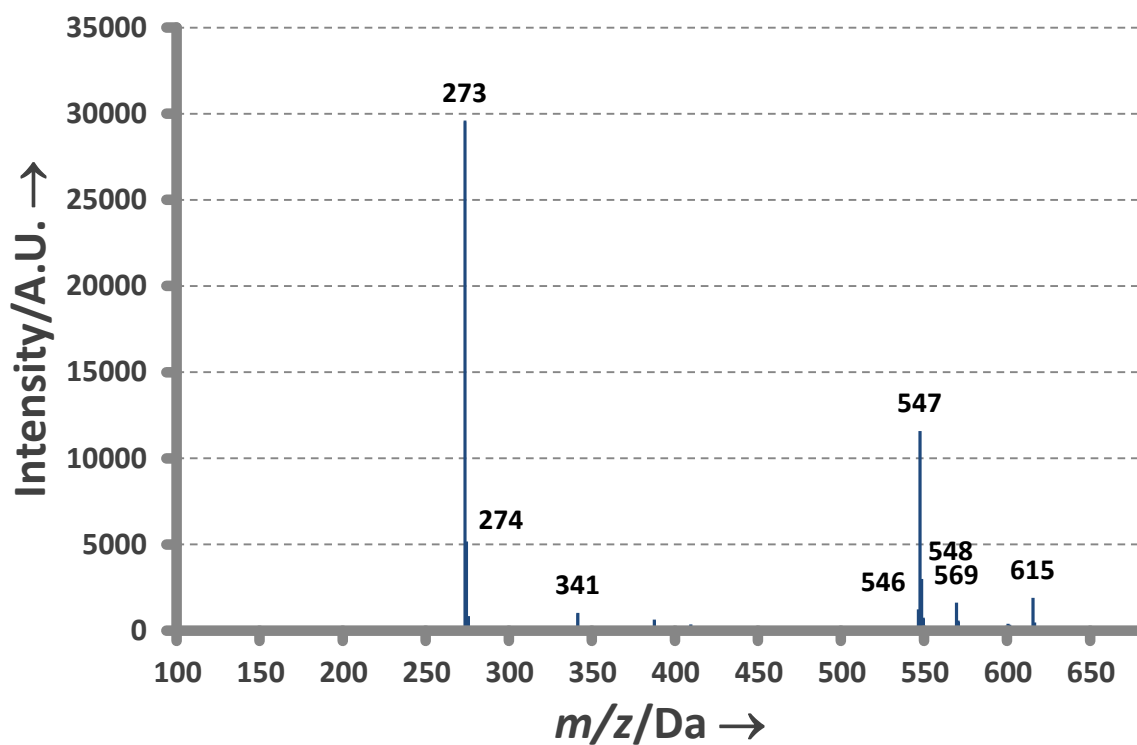


Figure S21 - Mass spectrum for chemically synth. dimer 2 at retention time 11.8 min (LC1).

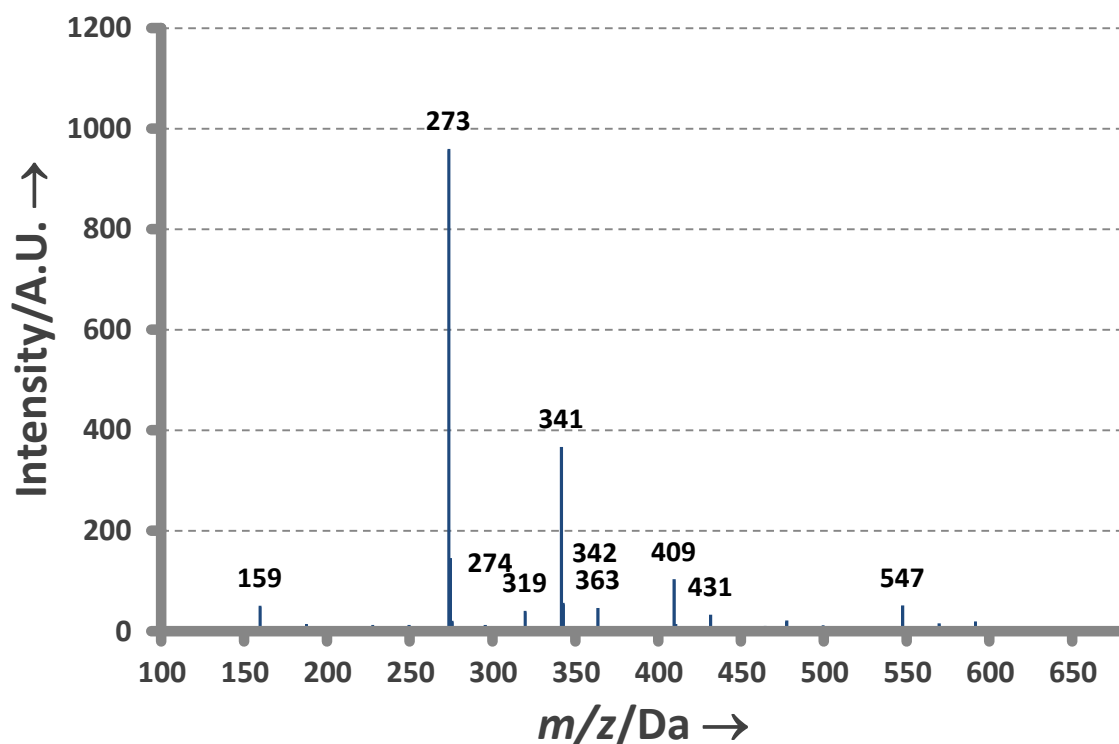


Figure S22 - Mass spectrum for chemically synthesized dimer **3** at retention time 7.7 min (LC3).

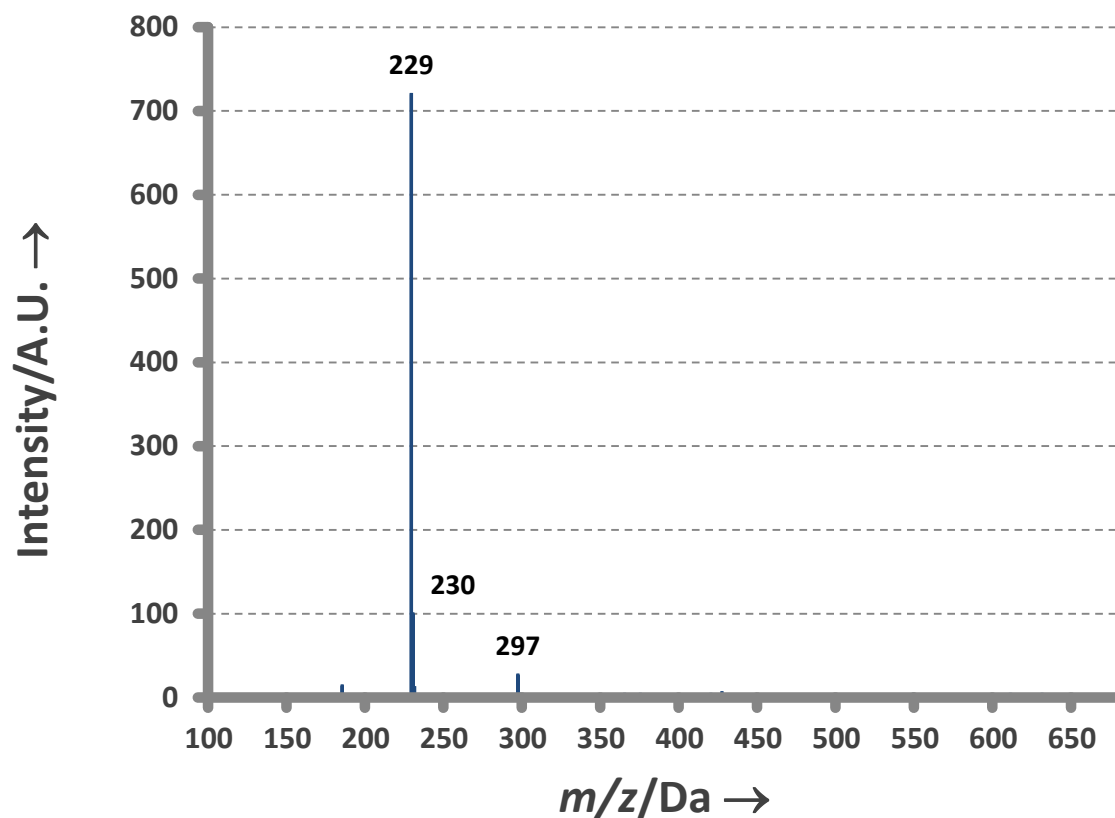
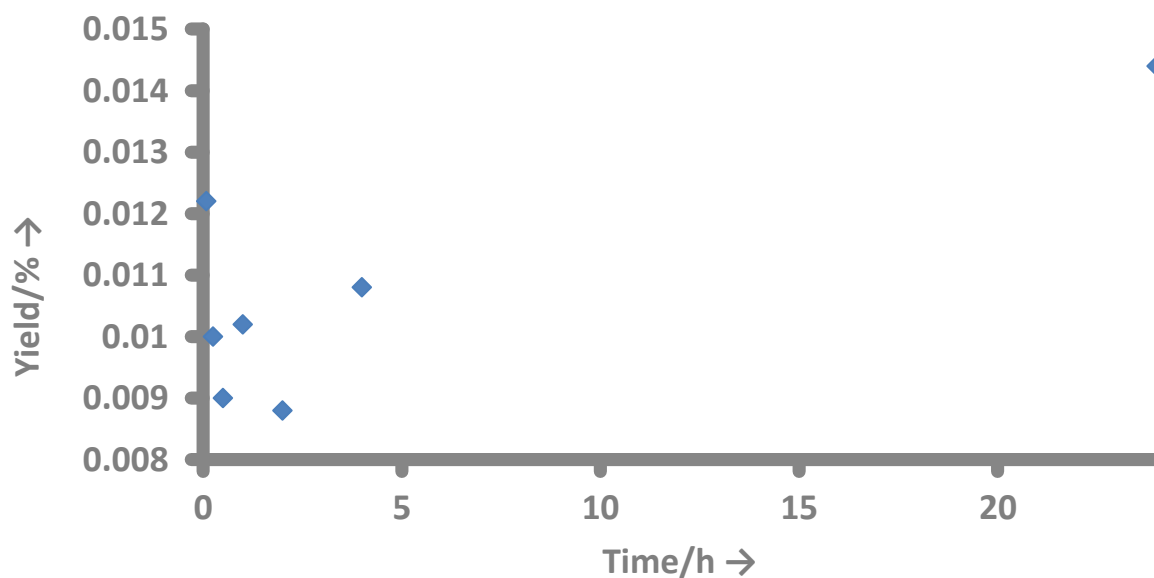
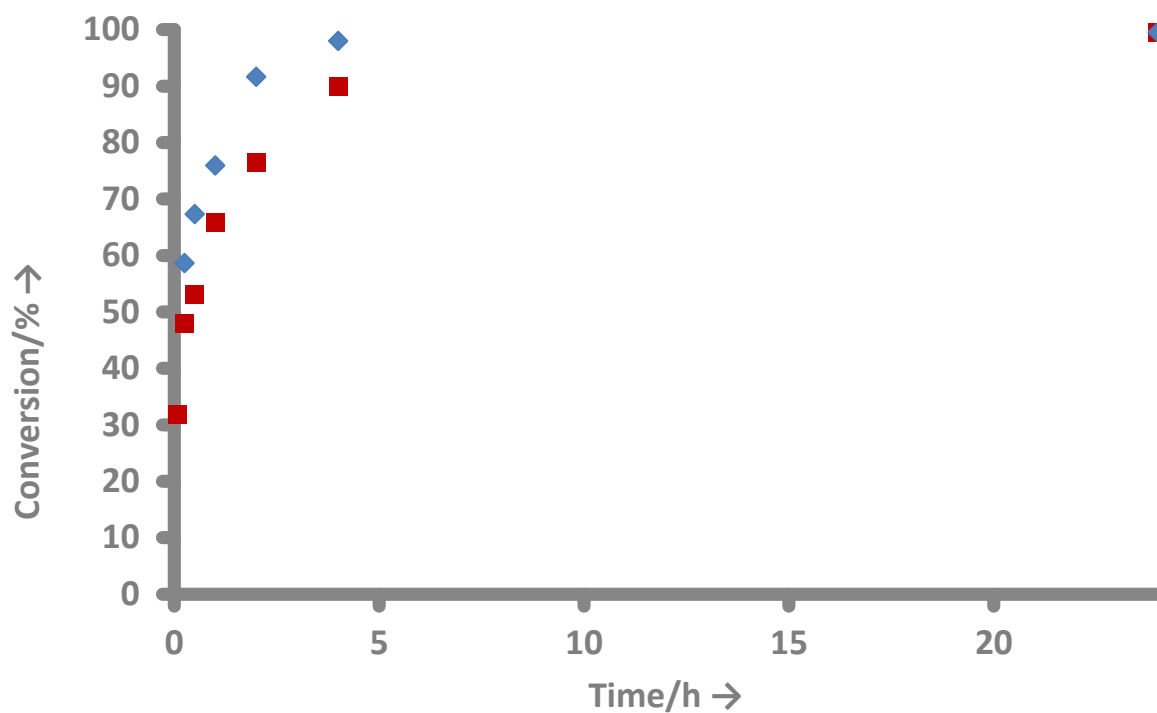


Figure S23 - Yield of dimer **3** in the laccase-mediated oligomerization of 4-HBA over time.



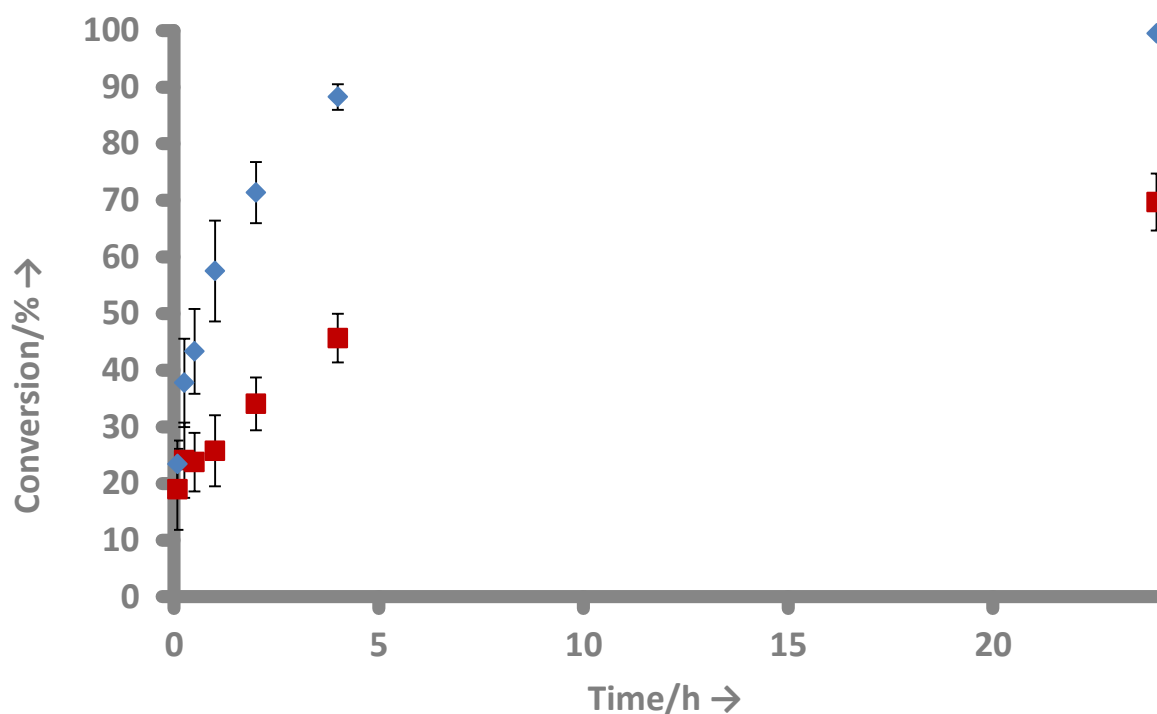
4-hydroxybenzoic acid (18.5 mg, 0.13 mmol, 26.8 mM) and laccase (4.8 U, 1.0 U ml⁻¹) were reacted in a 0.02 M ammonium acetate/acetic acid buffer (pH 5, 5.0 ml). The presence of dimer **3** was monitored at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h and 24 h by determination of the UV intensity corresponding to the peak of dimer **3** after LC-separation and interpolation on the respective calibration curve (SI figure S4).

Figure S24 - Conversion of dimer 3 by laccase over time.



Dimer **3** (1.6 mg, 6.8 μmol , 3.40 mM) only (blue diamonds) or in the presence of 4-hydroxybenzoic acid (red squares, 0.9 mg, 6.8 μmol , 3.40 mM) was reacted in the presence of laccase (0.51 U ml⁻¹) in a mixture of methanol and a 0.02 M ammonium acetate/acetic acid buffer (1:4.4) at room temperature and pH 5. The conversion of dimer **3** was monitored at 5 min (not for the reaction with dimer **3** only), 15 min, 30 min, 1 h, 2 h, 4 h and 24 h by determination of the UV intensity corresponding to the peak of dimer **3** after LC-separation and interpolation on the respective calibration curve (SI figure S4).

Figure S25 - Conversion of dimer 4 by laccase over time.



Dimer 4 only (blue diamonds, 4.2 mg, 18.1 μmol , 3.40 mM) or in the presence of 4-hydroxybenzoic acid (red squares, 2.5 mg, 18.1 μmol , 3.40 mM) was reacted in the presence of laccase (0.51 U ml^{-1}) in a mixture of methanol and a ammonium acetate/acetic acid buffer (1:4.4) at room temperature and pH 5. The conversion of dimer 4 was monitored at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h and 24 h by determination of the UV intensity corresponding to the peak of dimer 4 after LC-separation and interpolation on the respective calibration curve (SI figure S5). The experiment was performed in triplicate except for time point 1 (5 min) which was conducted in duplicate. Standard deviations over the whole population are included for every time point.

Figure S26 - ¹H NMR spectrum of methylated precursor of dimer 1 (solvent: CDCl₃).

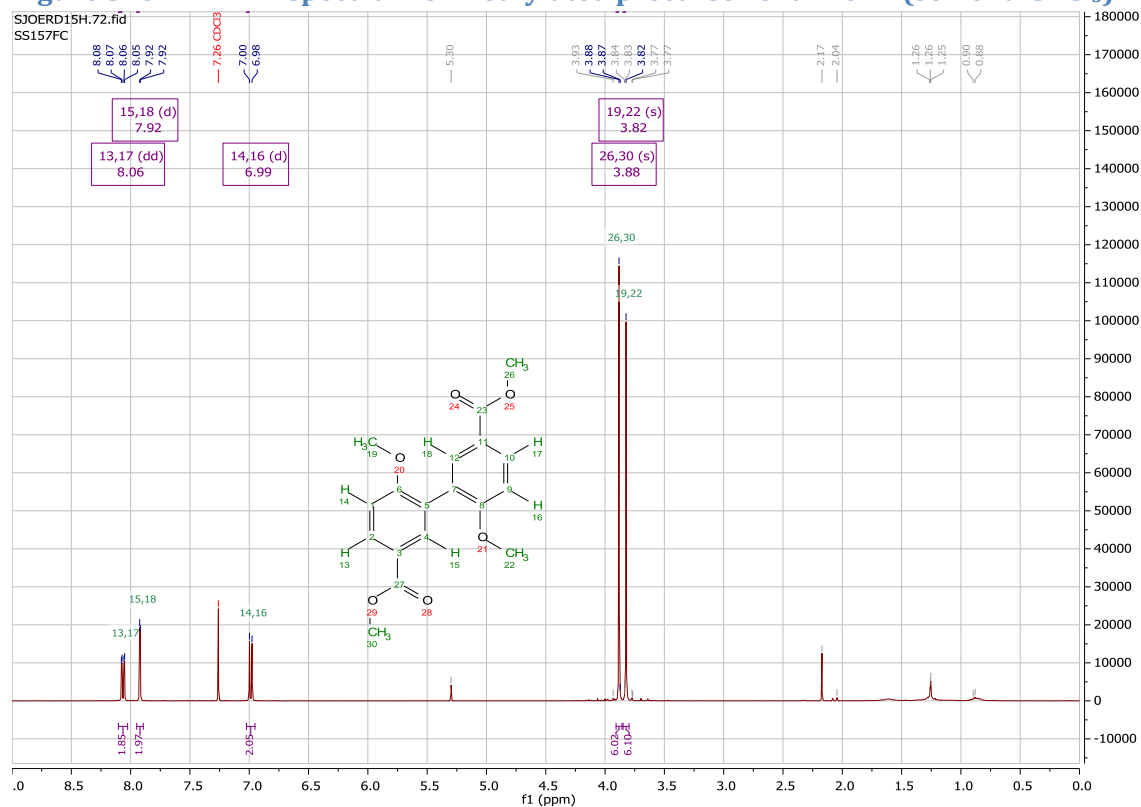


Figure S27 - ¹H NMR spectrum of methylated precursor of dimer 2 (solvent: CDCl₃).

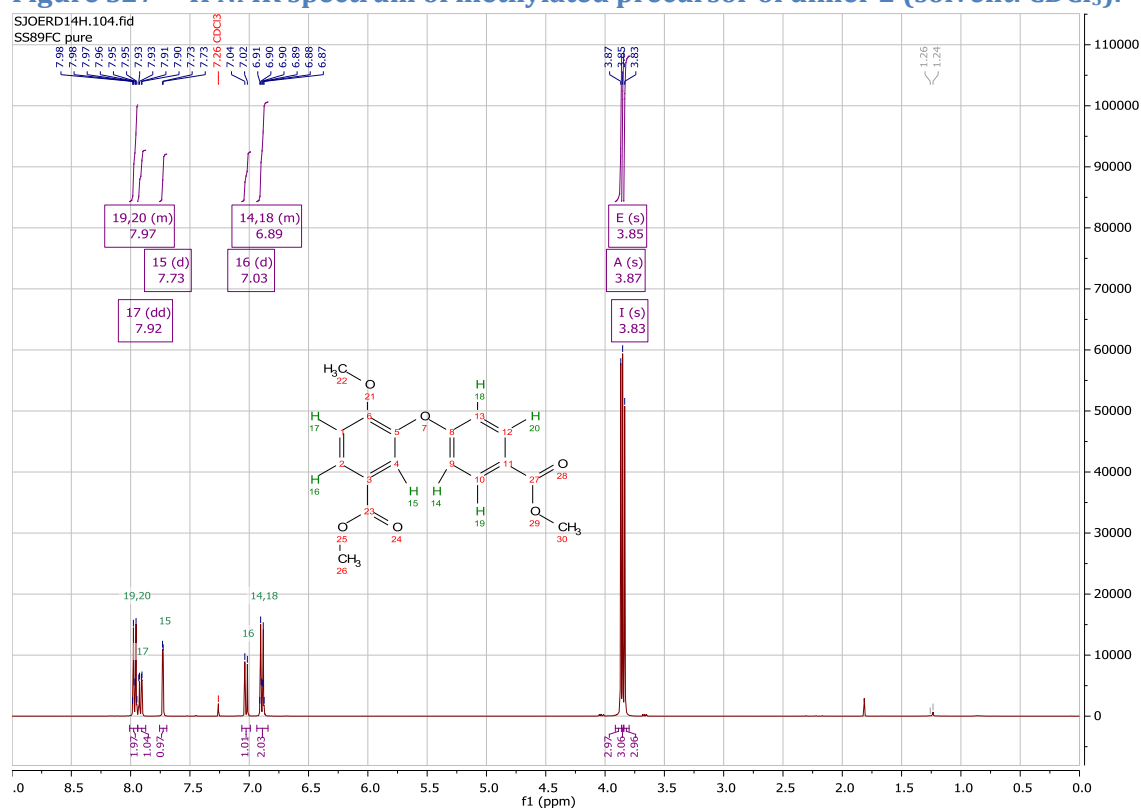


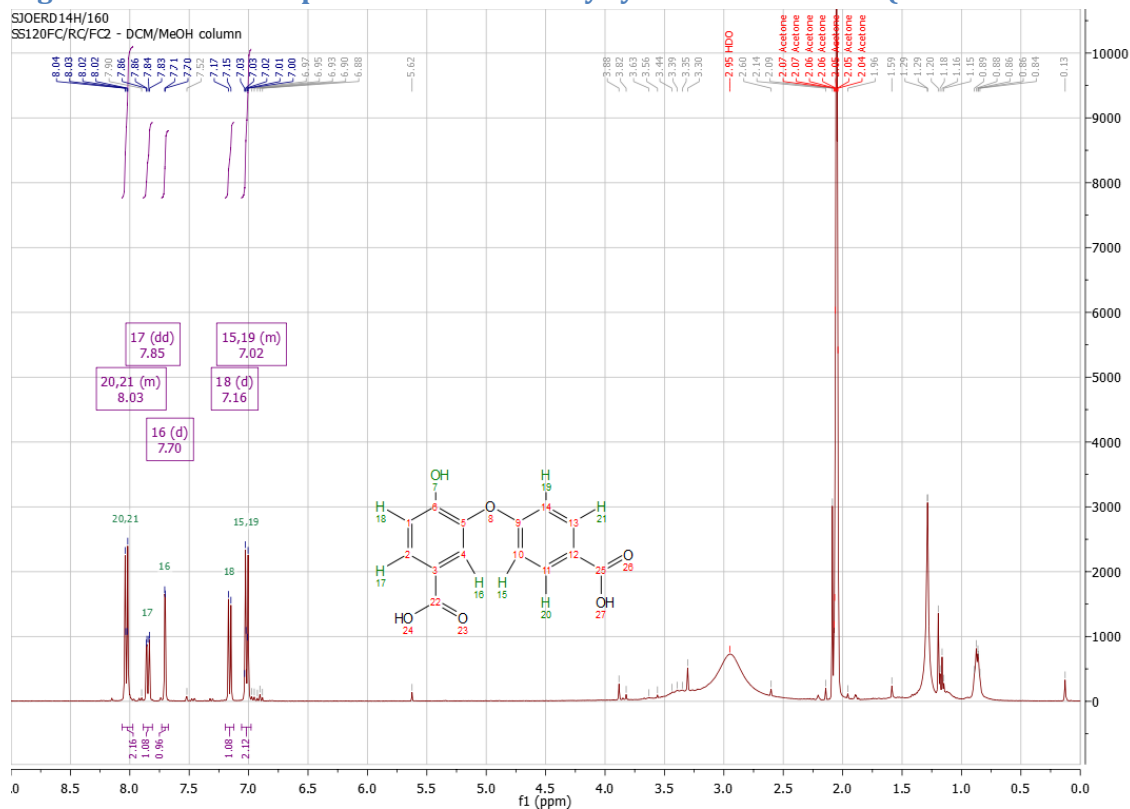
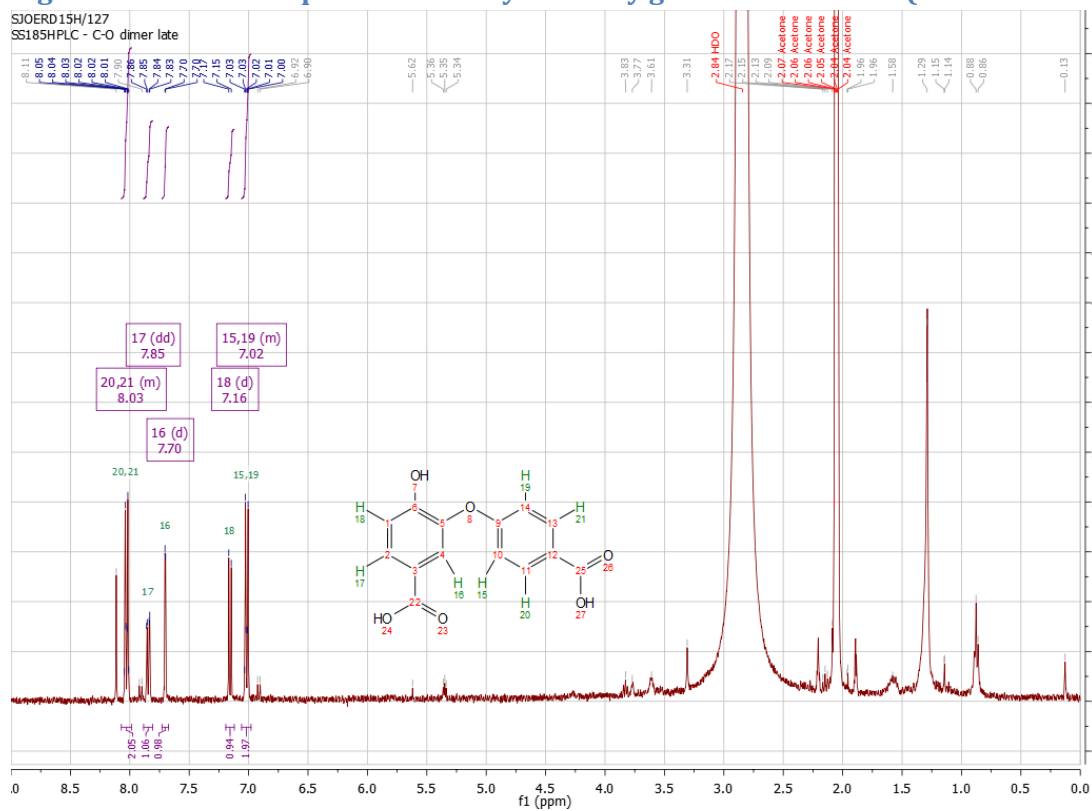
Figure S28 - ^1H NMR spectrum of chemically synthesized dimer 1 (solvent: acetone- d_6).Figure S29 - ^1H NMR spectrum of enzymatically generated dimer 1 (solvent: acetone- d_6).

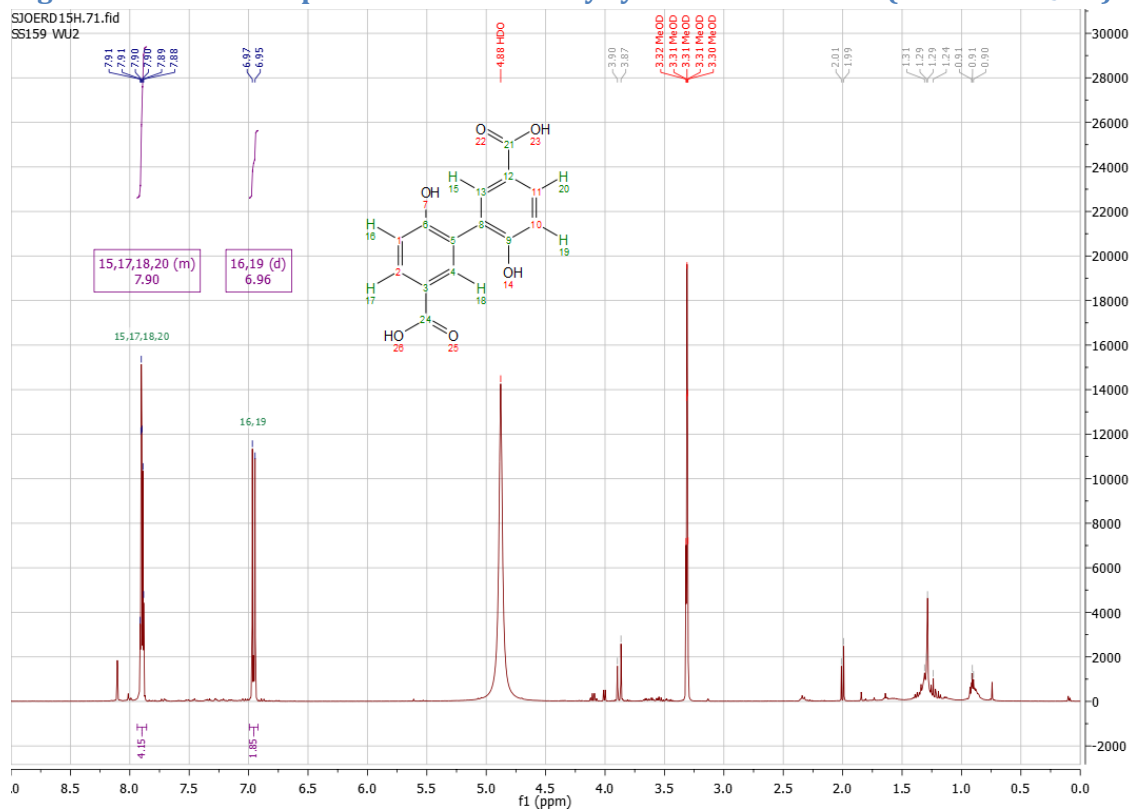
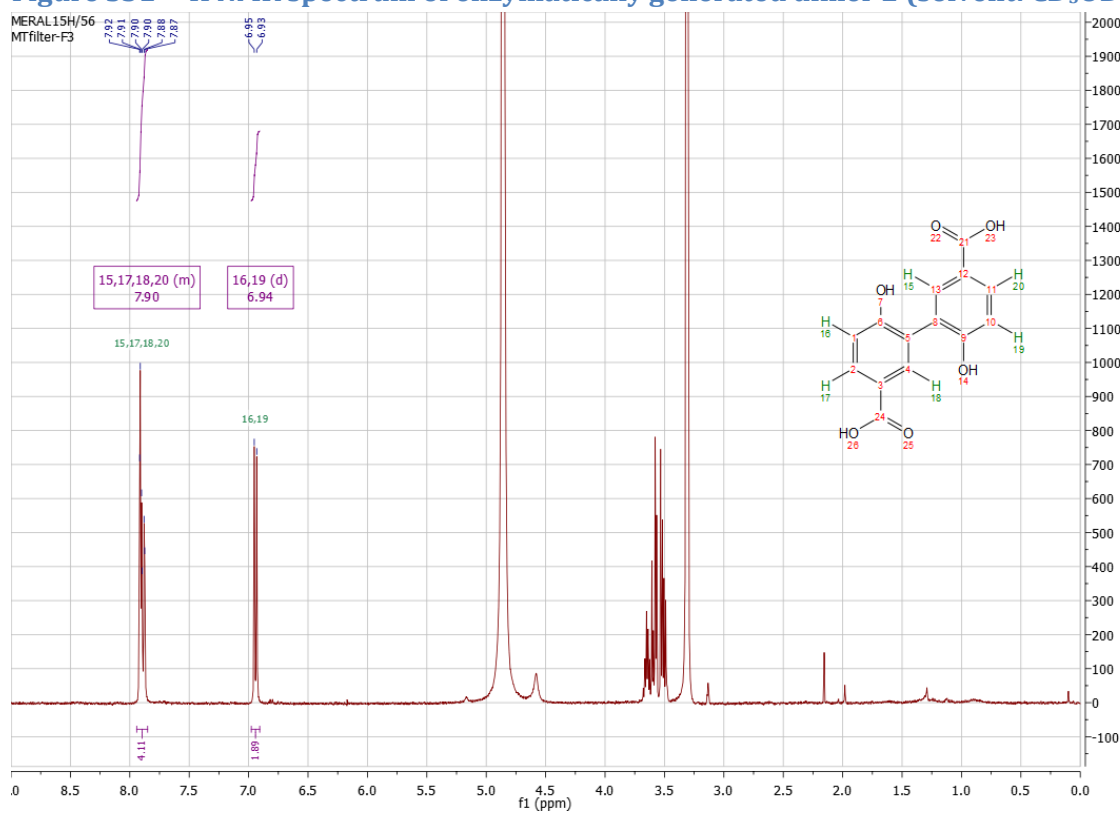
Figure S30 - ^1H NMR spectrum of chemically synthesized dimer 2 (solvent: CD_3OD).Figure S31 - ^1H NMR spectrum of enzymatically generated dimer 2 (solvent: CD_3OD).

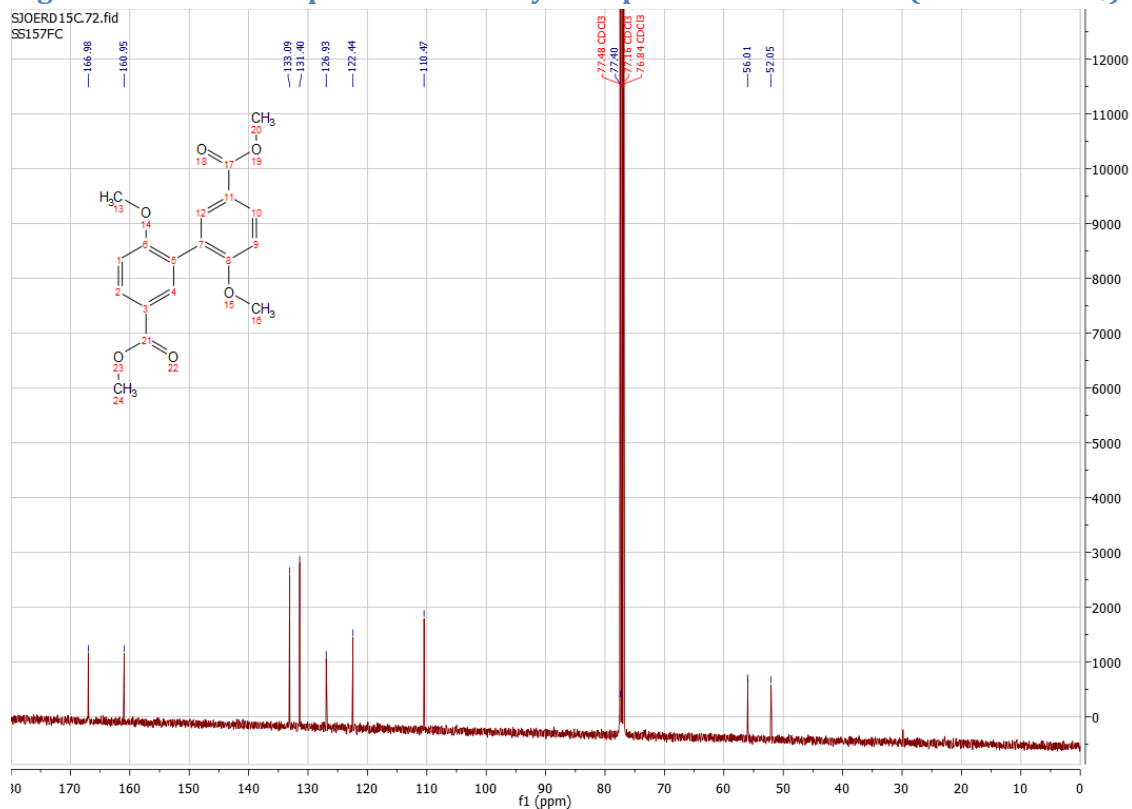
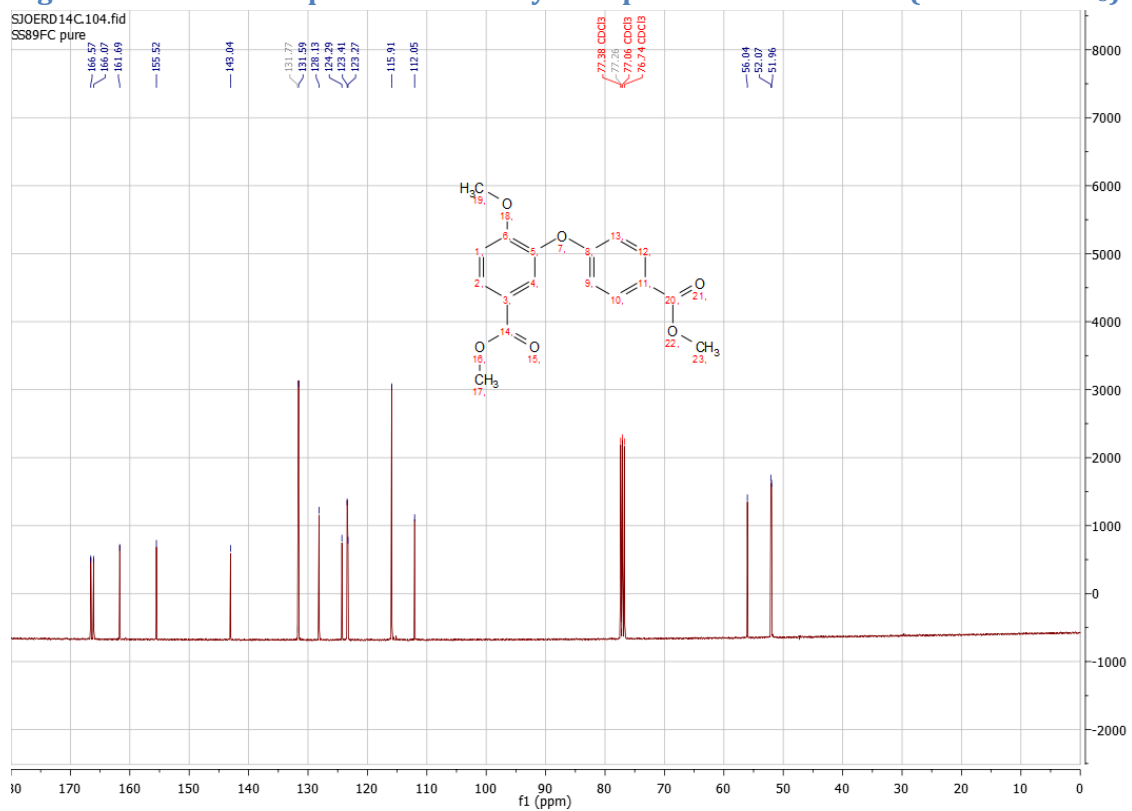
Figure S32 - ^{13}C NMR spectrum of methylated precursor of dimer 1 (solvent: CDCl_3).Figure S33 - ^{13}C NMR spectrum of methylated precursor of dimer 2 (solvent: CDCl_3).

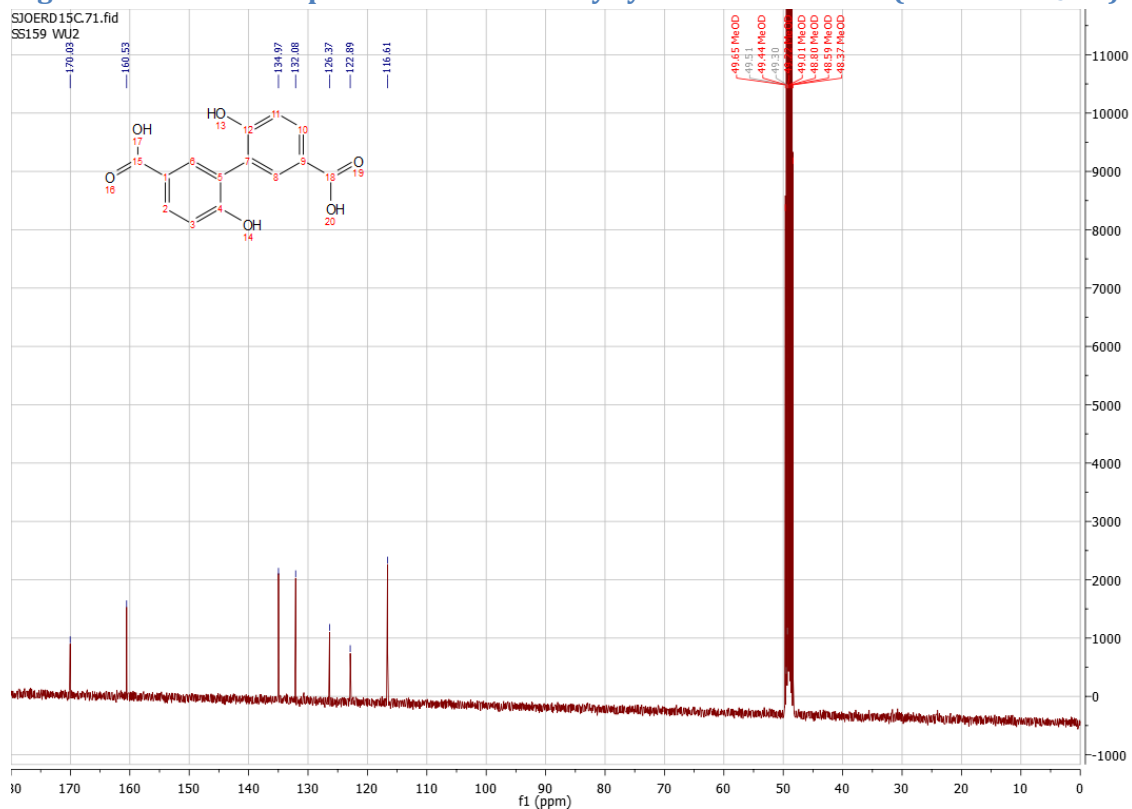
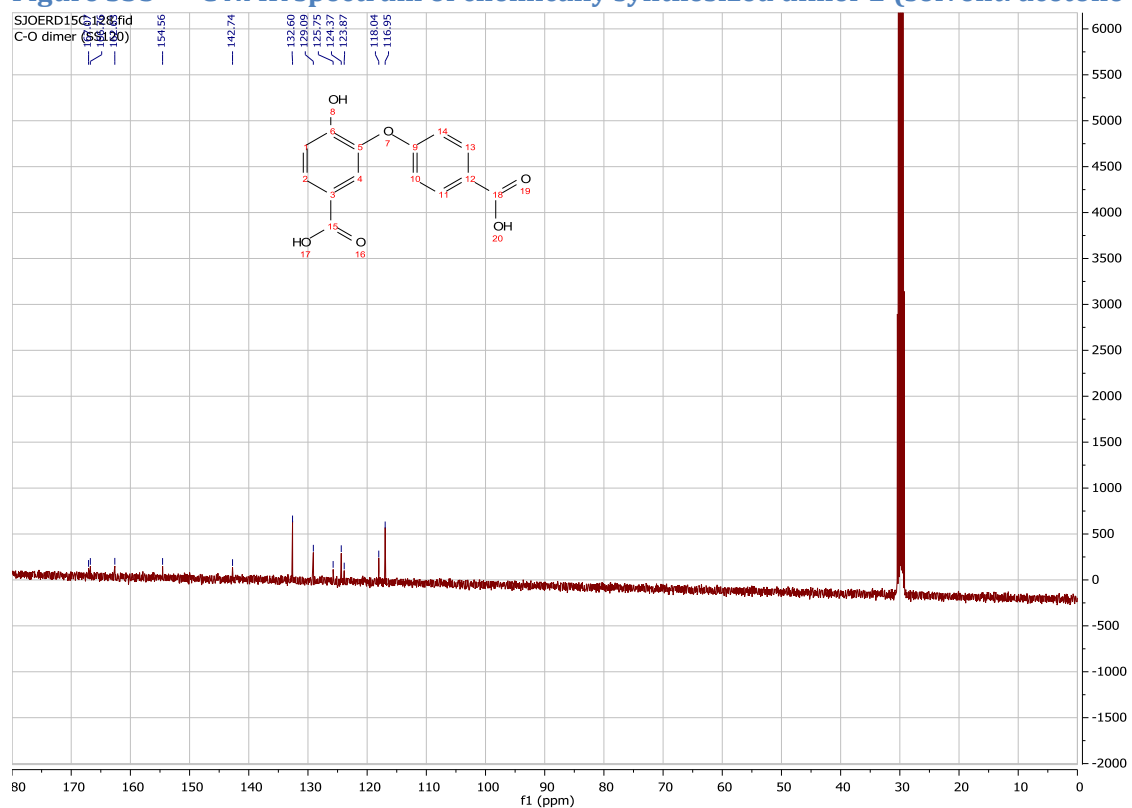
Figure S34 - ^{13}C NMR spectrum of chemically synthesized dimer 1 (solvent: CD_3OD).Figure S35 - ^{13}C NMR spectrum of chemically synthesized dimer 2 (solvent: acetone- d_6).

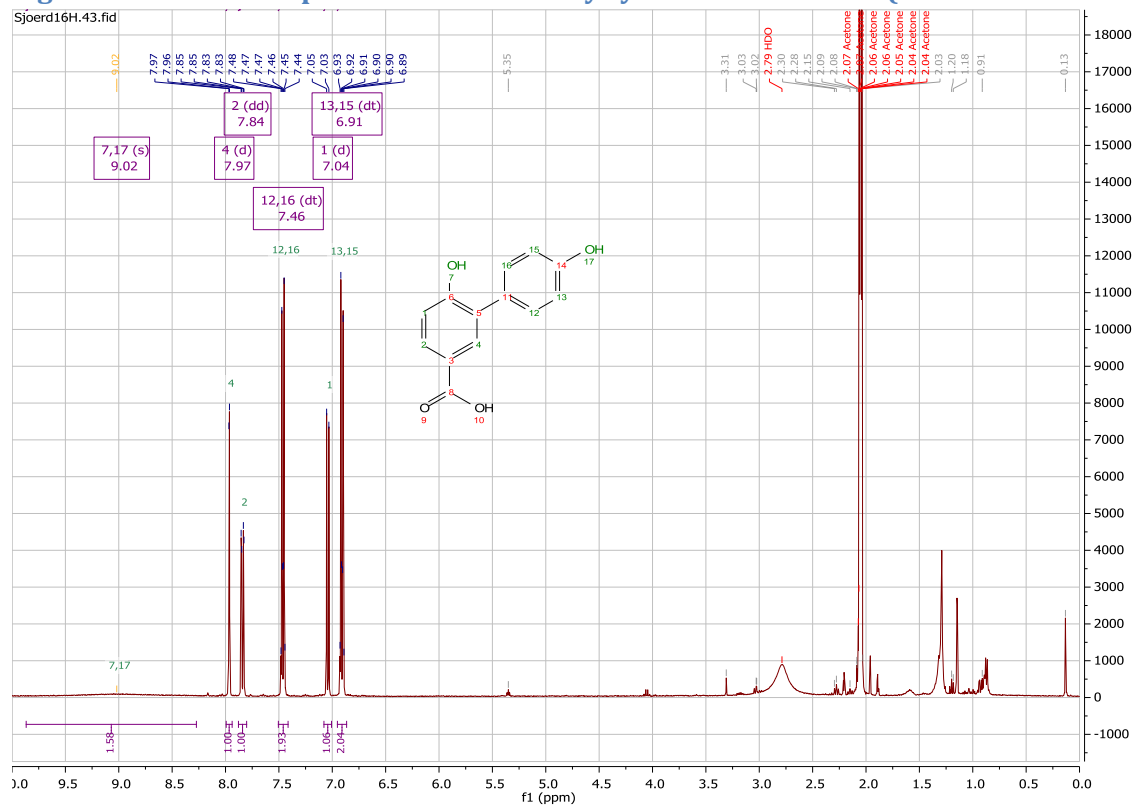
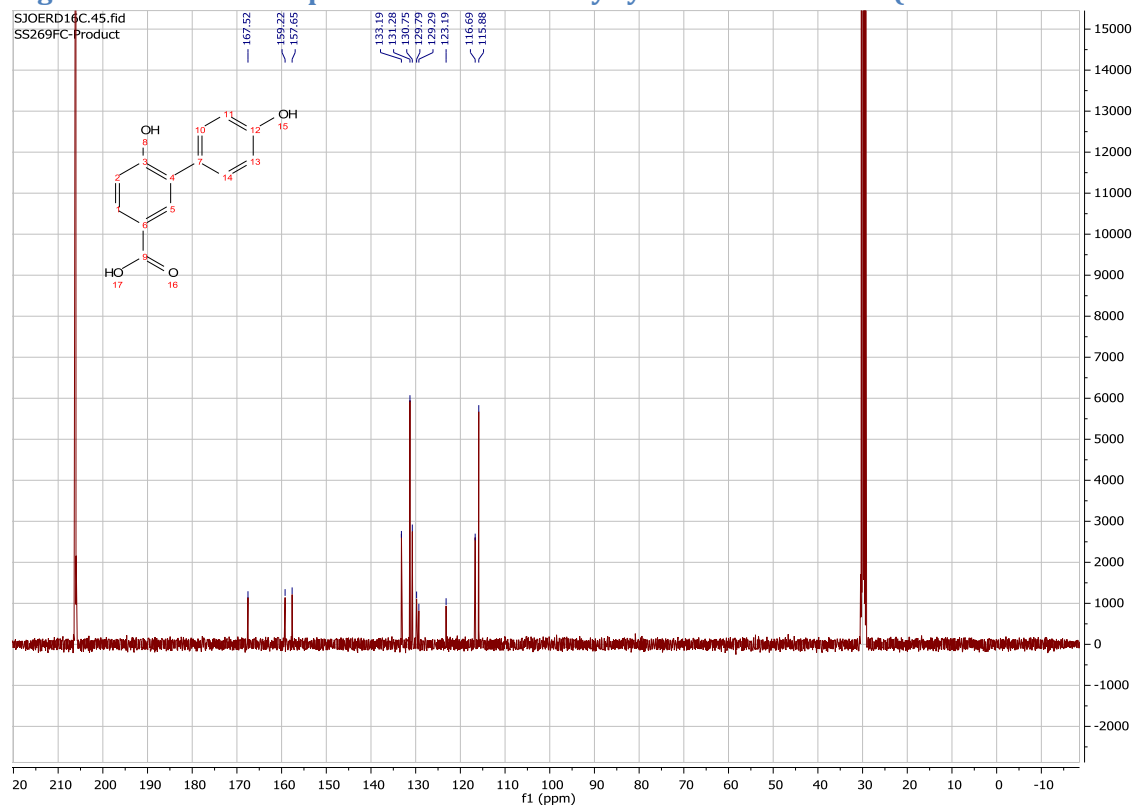
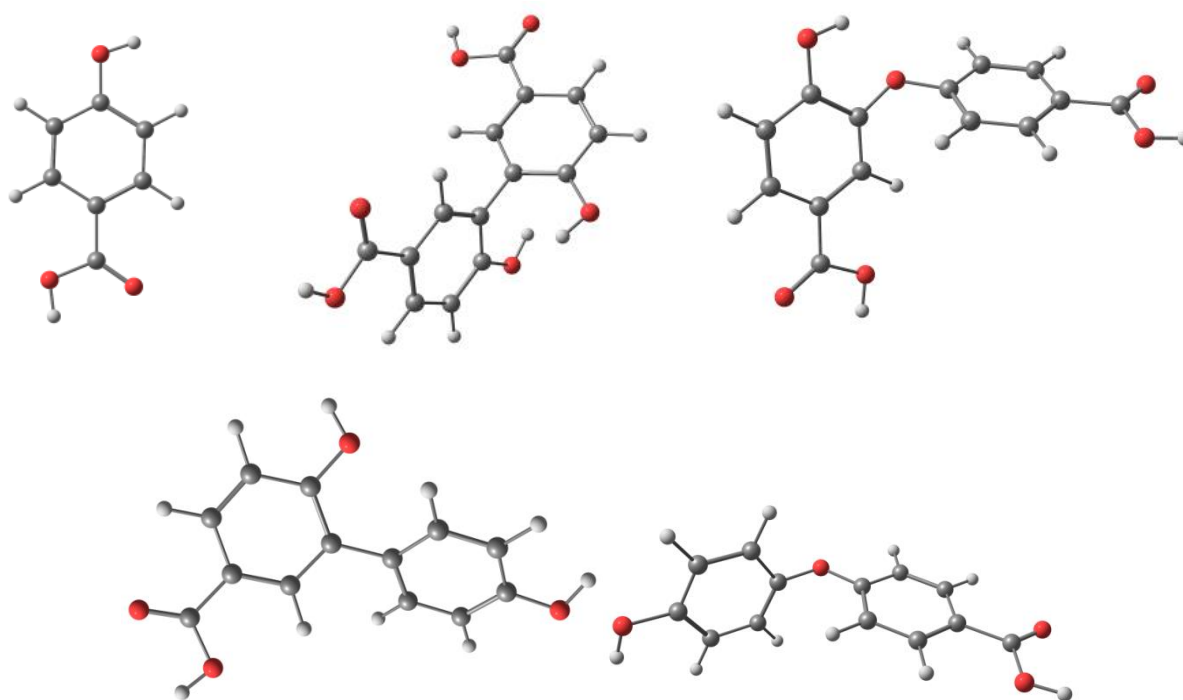
Figure S36 - ^1H NMR spectrum of chemically synthesized dimer 3 (solvent: acetone- d_6).Figure S37 - ^{13}C NMR spectrum of chemically synthesized dimer 3 (solvent: acetone- d_6).

Figure S38 - Optimized geometries for 4-hydroxybenzoic acid, dimer 1 and dimer 2.

Optimized geometries obtained through quantum chemical calculations at the M11L level (6-311+G(d,p)) in water. Upper left) 4-hydroxybenzoic acid, upper middle) dimer **1**, upper right) dimer **2**, lower left) dimer **3** and lower right) dimer **4**.

Table S1 - Calculated relative energies for the formation of the radical cation.

Table S1. Calculated relative energies for the formation of the radical cation from 4-hydroxybenzoic acid and dimers corresponding to the geometries displayed in SI figure S38.

Level (solvation) ^[a]	Energy gap between substrate and its radical cation ^[b]				
	4-HBA	Dimer 1	Dimer 2	Dimer 3	Dimer 4
M11L (water)	154.9	152.7	147.8	137.1	138.1
M11L (vacuum)	200.9	192.5	187.4	172.5	176.2
B3LYP (vacuum)	199.1	193.2	186.6	173.8	177.6

[a] Level of quantum chemical calculation with the 6-311+G(d,p) basis set. [b] Energies are given in kcal mol⁻¹.

Table S2 - Comparison of ¹H NMR data for dimers 1 and 2.

Table S2. Comparison of ¹H NMR data for 4-HBA dimers which have been chemically synthesized and dimers generated through the laccase-mediated reaction.

Dimer 1^[a]

Synthetic (δ/ppm)	Enzymatic (δ/ppm)
6.96 (d, ³ J(H,H) = 9.1 Hz, 2H)	6.94 (d, ³ J(H,H) = 8.4 Hz, 2H)
7.94 – 7.87 (m, 4H)	7.95 – 7.84 (m, 4H)

Dimer 2^[b]

Synthetic (δ/ppm)	Enzymatic (δ/ppm)
7.02 (d, ³ J(H,H) = 8.8 Hz, 2H)	7.06 – 6.98 (m, 2H)
7.16 (d, ³ J(H,H) = 8.5 Hz, 1H)	7.16 (d, ³ J(H,H) = 8.5 Hz, 1H)
7.70 (d, ³ J(H,H) = 2.0 Hz, 1H)	7.70 (d, ³ J(H,H) = 2.1 Hz, 1H)
7.85 (dd, ³ J(H,H) = 8.5, 2.0 Hz, 1H)	7.85 (dd, ³ J(H,H) = 8.5, 2.1 Hz, 1H)
8.03 (d, ³ J(H,H) = 8.8 Hz, 2H)	8.07 – 7.98 (m, 2H)

[a] NMR solvent = methanol-*d*₄. [b] NMR solvent = acetone-*d*₆.