**Supplemental Material**

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**Phenolic metabolism in the hornwort *Anthoceros agrestis*: 4-Coumarate CoA Ligase and 4-hydroxybenzoate CoA ligase**

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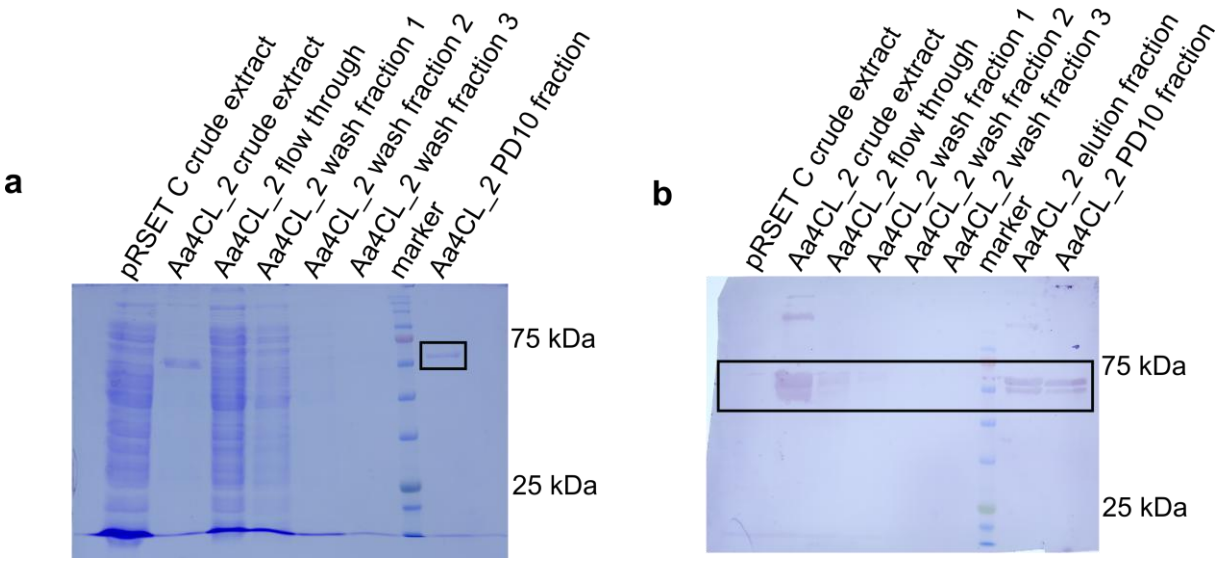
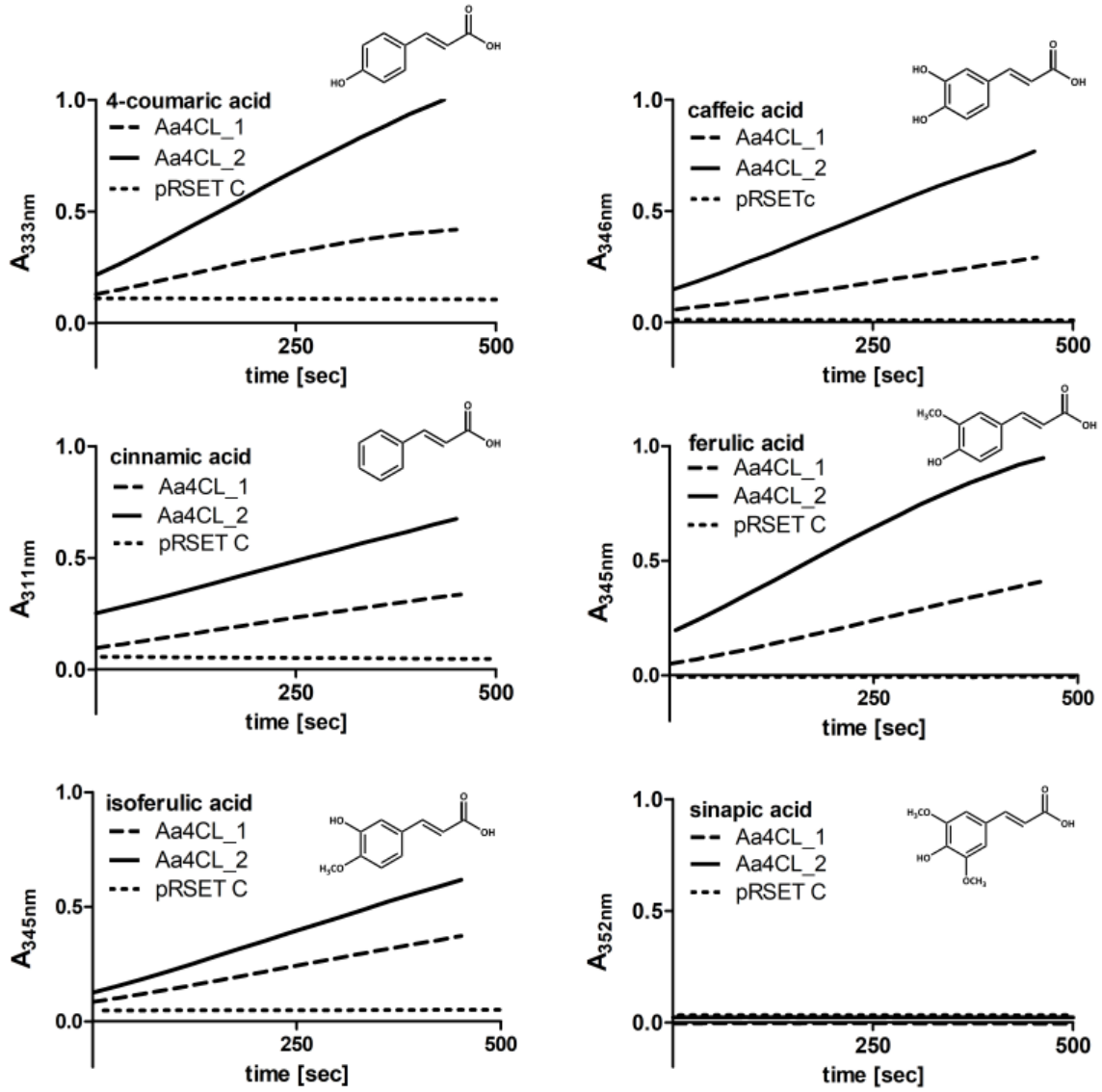
**Suppl. Table S1** List of PCR primers: binding sequence underlined and restriction sites marked red

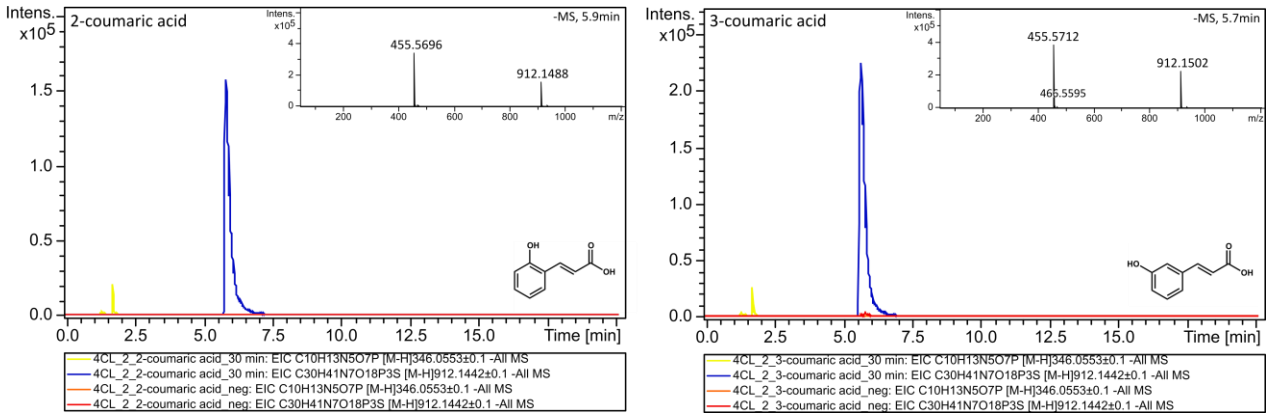
| Primer name | Sequence |
|---|---|
| AaAp6378_f | TTCCGCTCCAACTTGCCCG |
| Aa Ap6378_r | ATCCTCCAGCTCCTTCCCC |
| Aa Ap6378_5´R | CTCGACACCACCTCCACCTCGCTGTAG |
| Aa Ap6378_3´R | GTGATGCTGTGCGGGCTGCGTGCT |
| Aa Ap6378_fl1_f | TATCTGCAGATGGCGCCGATTCTTGCACCTCC |
| Aa Ap6378_fl2_f | TATCTGCAGATGCCTGCGGAGATGGAGGCC |
| Aa Ap6378_fl_r | TATAAGCTTCTACACCCTGCTCCTCAGCTCC |
| Aa35279_f | TCTTCGGGGACAACAGGACTG |
| Aa35279_r | AACCTGGAACCCTTTGTACTTGATG |
| Aa35279_ 5´R | CCGTTGTGCCGTAAATGTGGAAGAAGGGC |
| Aa35279_3´R | GGAAAGTCCCTGCCTGCAAATCGTG |
| Aa35279_fl_f | TATCTCGAGATATGGCCTCTCTCTCCGAGCC |
| Aa35279_fl_r | TATAAGCTTTCAAATTAGTTGGTTCTCAGTCTTCTTGAG |



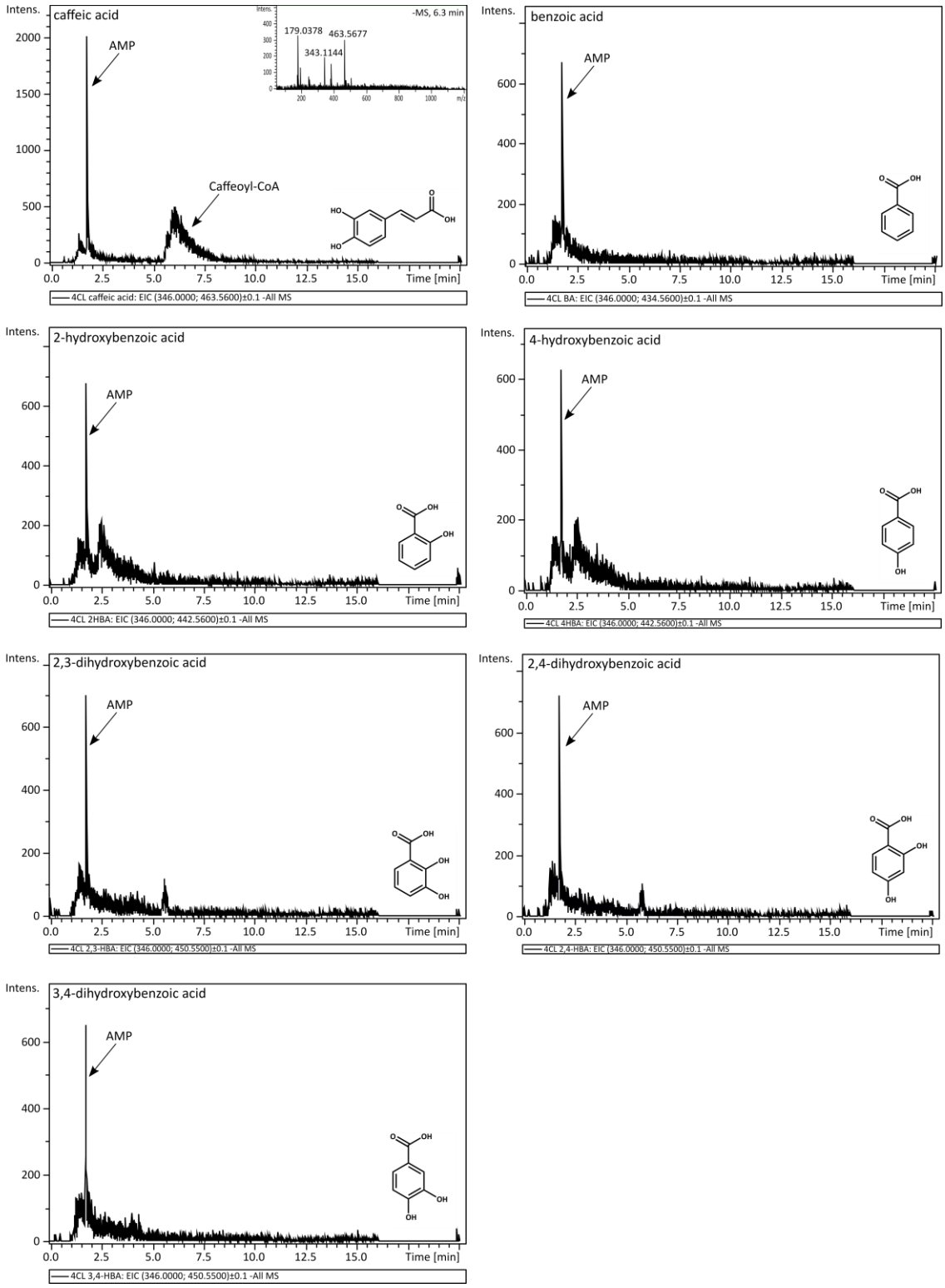

**Suppl. Fig. S1** SDS-Page and Western blot of Aa4CL_2 heterologously expressed in *E. coli* SoluBL21. (a) Coomassie stained SDS-PAGE gel of heterologously expressed Aa4CL_2 with *N*-terminally attached 6xHis-tag before and after purification by metal chelate chromatography and equally treated crude protein extract from *E. coli* SoluBL21 carrying the empty vector pRSET C. (b) Western blot after detection using anti-6x-His-tag monoclonal mouse antibody and goat anti-mouse antibody conjugated to alkaline phosphatase and NBT/BCIP colour reaction



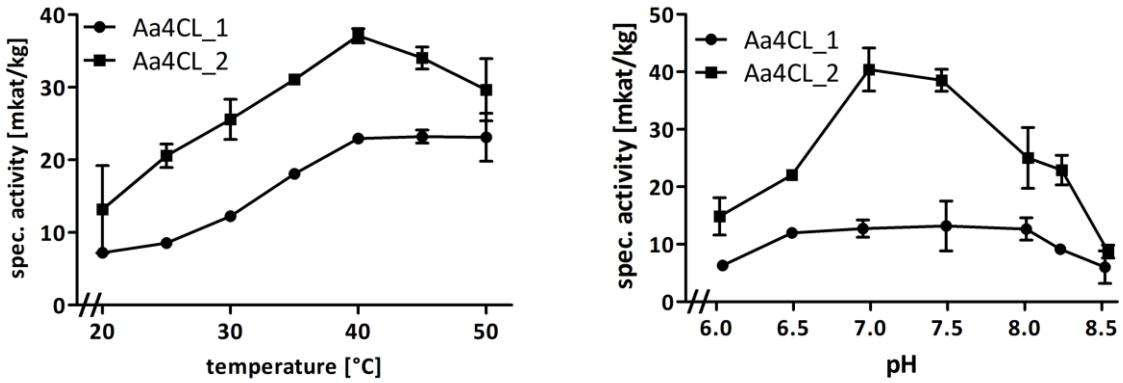

**Suppl. Fig. S2** Activity of purified Aa4CL_1 and Aa4CL_2 (3 µg protein) heterologously expressed in *E. coli* with different hydroxycinnamic acid substrates (500 µM). Crude protein extract (60 µg protein) from transformed *E. coli* with the empty vector pRSET C served as a negative control




**Suppl. Fig. S3** LC-MS analysis of CoA esters formed by Aa4CL with 2- and 3-coumaric acid. Purified Aa4CL_2 (8 µg protein) was incubated with 500 µM substrate, 1.25 mM ATP and 1 mM CoA for 30 min at 40 °C. Heat-denatured protein (10 min 95 °C) was used as a negative control. The chromatograms show the EIC of the expected products AMP (m/z 346.0553 ± 0.1) in yellow for Aa4CL_2 and orange for the negative control. The corresponding CoA-ester is displayed in blue for Aa4CL_2 or red for the negative control. The exact mass of the resulting CoA-ester is shown in each chromatogram. For all produced CoA-esters we observed the [M-H] pseudo molecular ion (m/z 912.15) as well as the doubly charged molecular ion (m/z 455.57) [M/2]-H

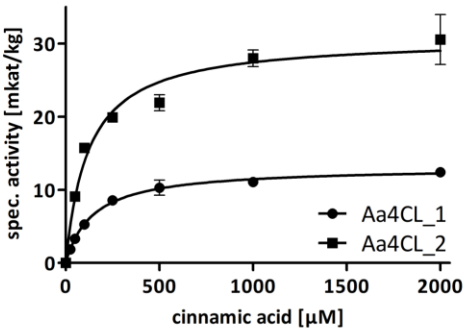


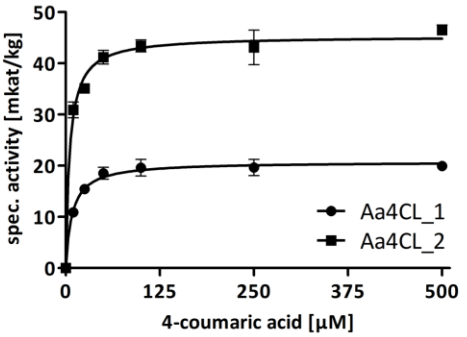

**Suppl. Fig. S4** LC-MS analysis of CoA esters formed by Aa4CL with various (hydroxy)benzoic acids and caffeic acid. Purified Aa4CL_2 (7.5 µg protein) was incubated with 500 µM caffeic, benzoic, 2-hydroxybenzoic, 4-hydroxybenzoic, 2,3-dihydroxybenzoic, 2,4-dihydroxybenzoic or 3,4-dihydroxybenzoic acids for 1 h at 35 °C. The chromatograms show the EIC of the expected products AMP (m/z 346 ± 0.1) and the corresponding CoA-ester (doubly charged molecular ion [M/2]-H) of caffeic acid (m/z 463.56 ± 0.1), benzoic acid (m/z 434.56 ± 0.1), monohydroxylated benzoic acids (m/z 442.56 ± 0.1) or dihydroxylated benzoic acids (m/z 450.55 ± 0.1)

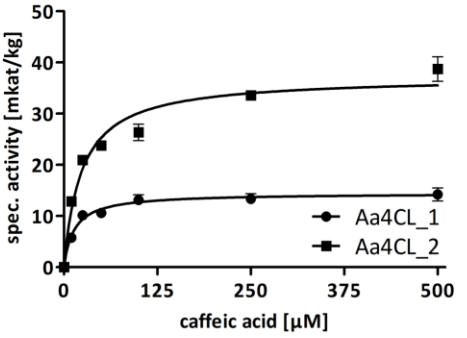


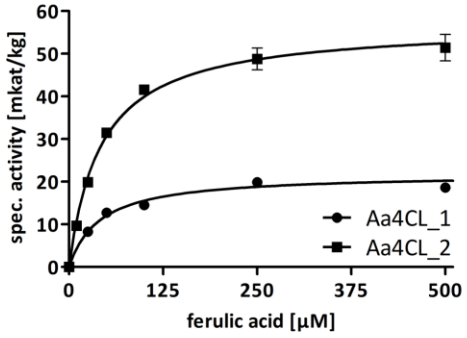

**Suppl. Fig. S5** Temperature (left) and pH-optimum (right) of Aa4CL_1 and Aa4CL_2 (mean ± SD, n=7)

## Michaelis-Menten

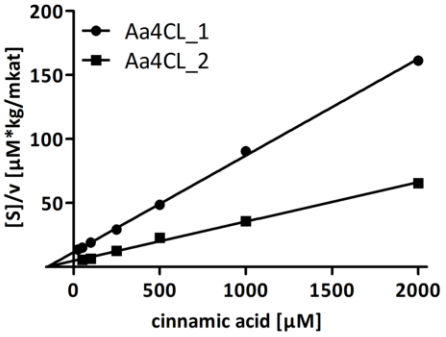


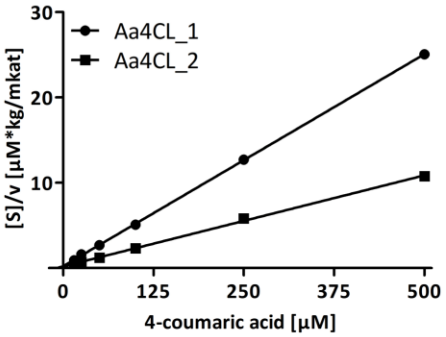



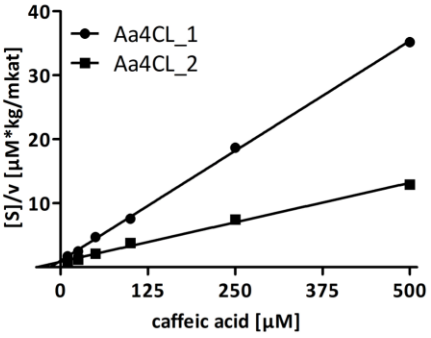



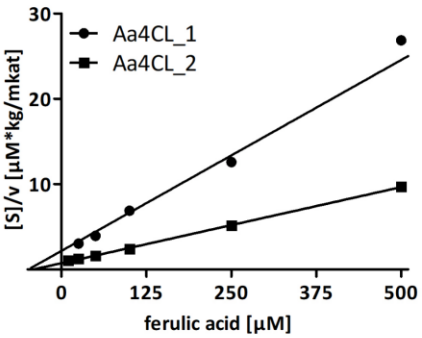



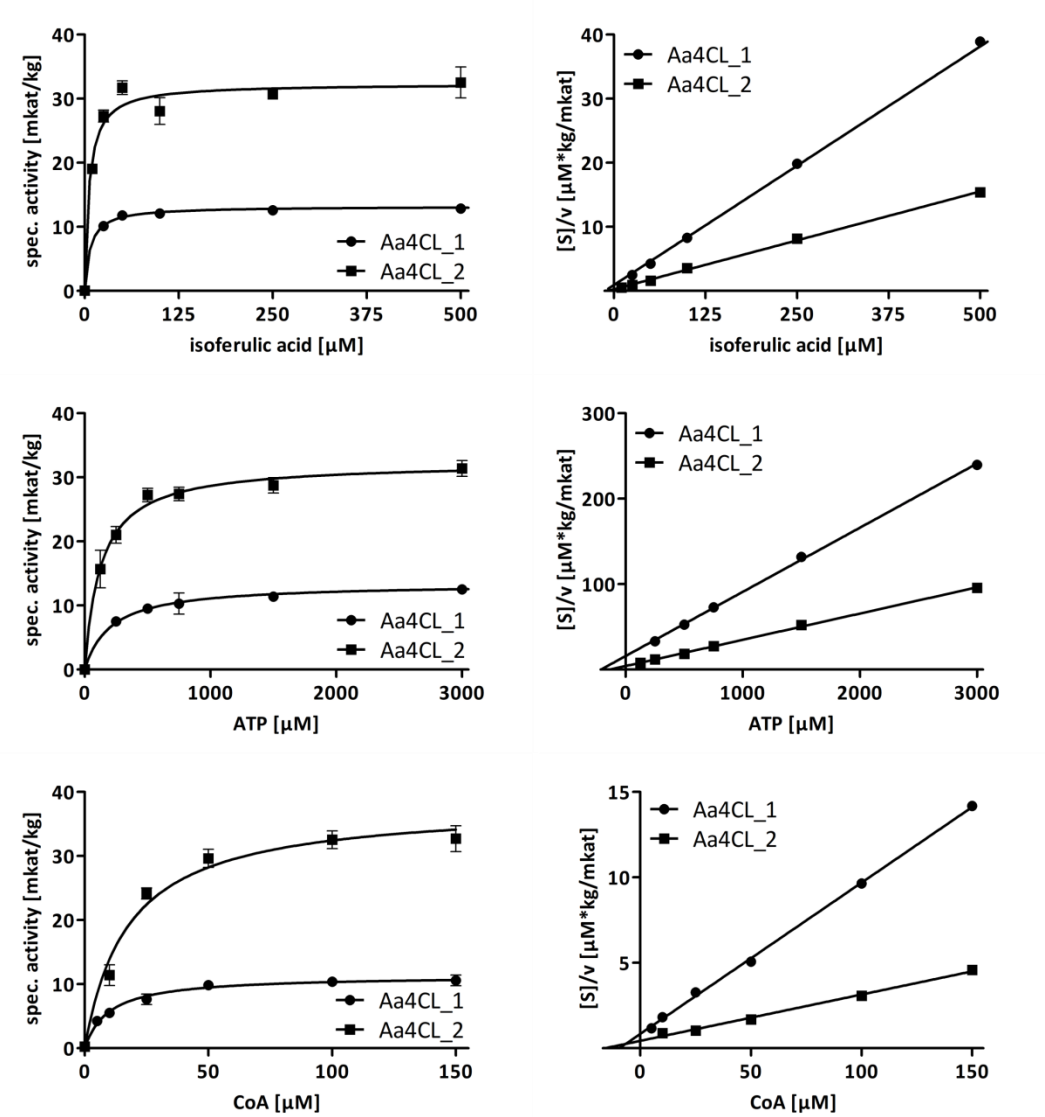

## Hanes-Woolf



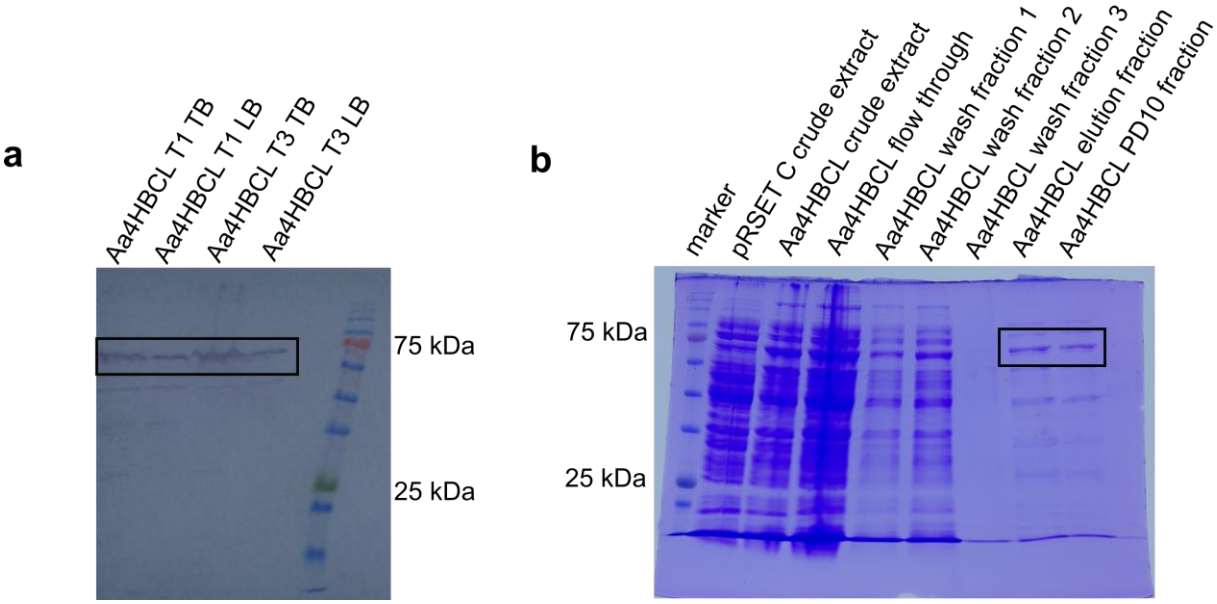



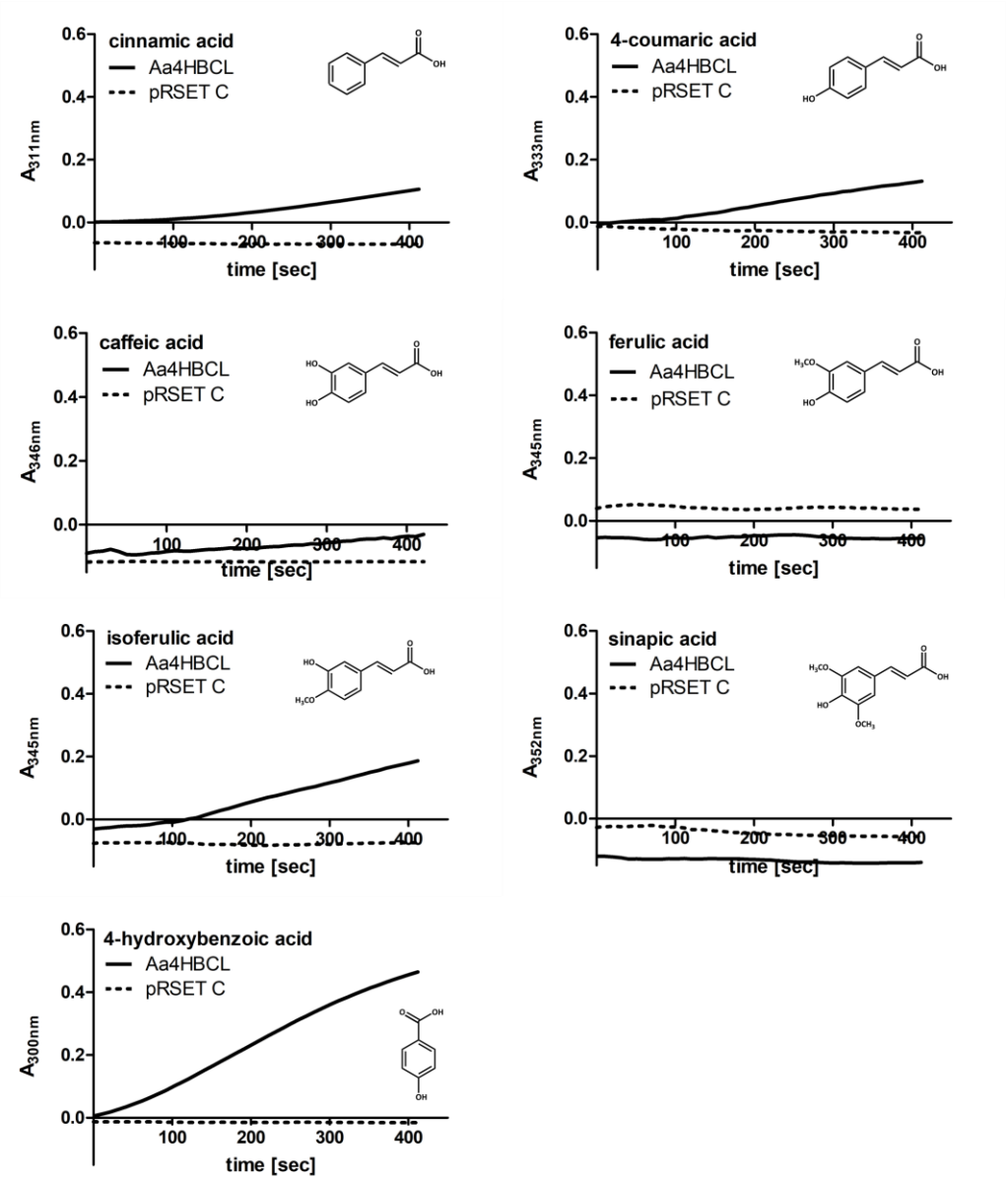





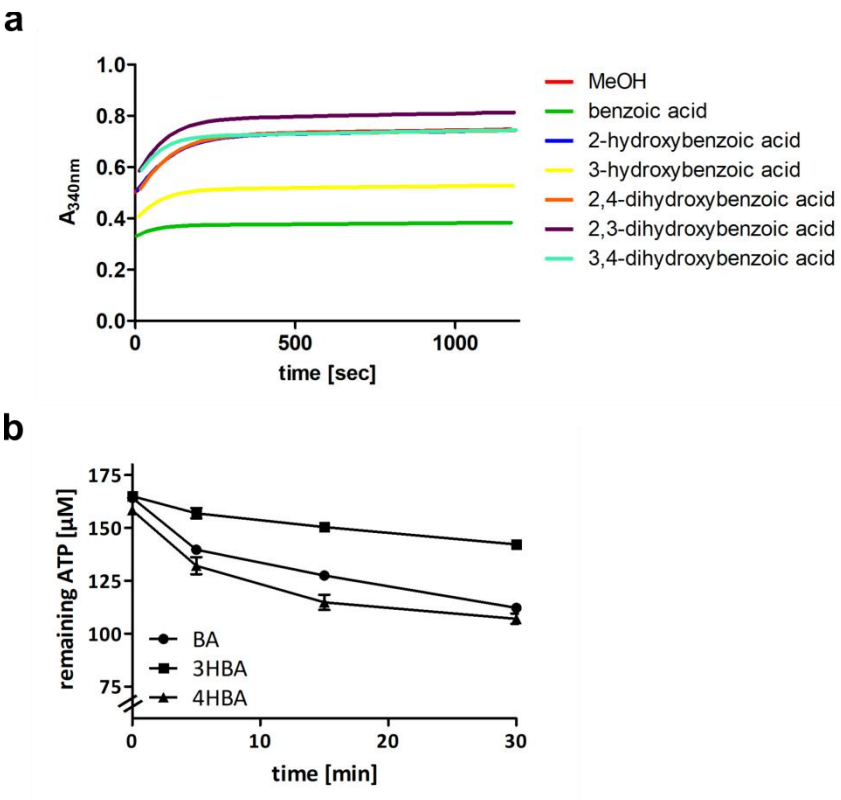

**Suppl. Fig. S6** Substrate saturation curves of reactions of Aa4CL_1 and Aa4CL_2 for the determination of $K_m$-values (mean ± SD, n = 9). Hydroxycinnamic acids were incubated with 2.5 mM ATP and 100 µM CoA. For the determination of $K_m$-values for ATP 100 µM CoA and for CoA 2.5 mM ATP were used together with 500 µM caffeic acid. Michaelis-Menten diagrams are shown on the left and Hanes-Woolf diagrams on the right



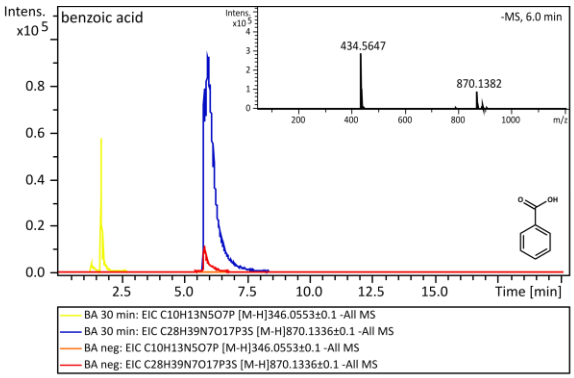

**Suppl. Fig. S7** Western blot and SDS-Page of Aa4HBCL heterologously expressed in *E. coli* SoluBL21. (a) Western blot analysis of His-tagged Aa4HBCL expressed in two different *E. coli* SoluBL21 transformants (T1 or T3) in either TB or LB media. (b) Coomassie stained SDS-PAGE gel of heterologously expressed Aa4HBCL with *N*-terminally attached 6xHis-tag before and after purification by metal chelate chromatography and equally treated crude protein extract from *E. coli* carrying the empty vector pRSET C

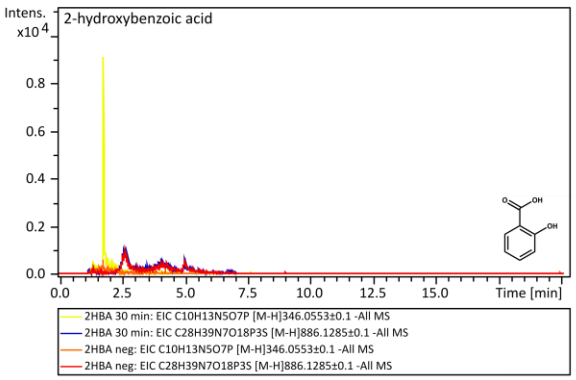


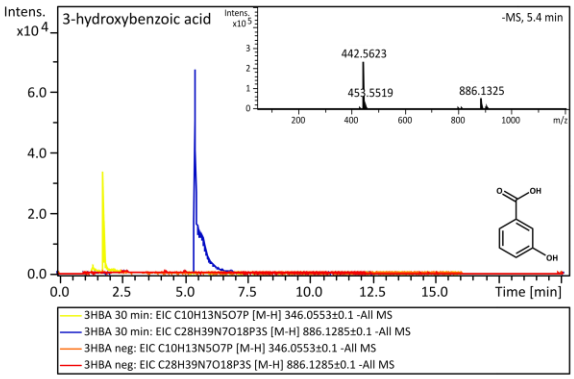

**Suppl. Fig. S8** Activity of heterologously expressed purified Aa4HBCL (32 µg protein) with 500 µM 4-hydroxybenzoic, cinnamic, 4-coumaric, caffeic, ferulic, isoferulic or sinapic acids. Crude protein extract (60 µg protein) from transformed *E. coli* with the empty vector pRSET C served as a negative control

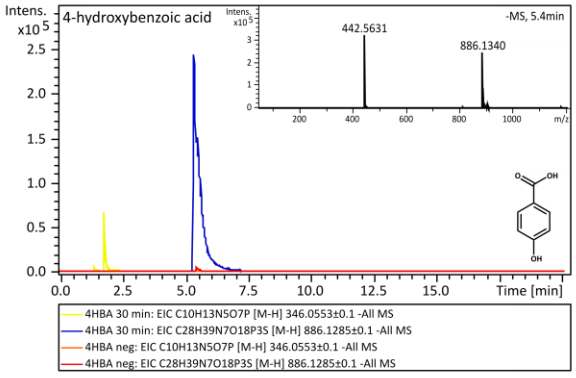


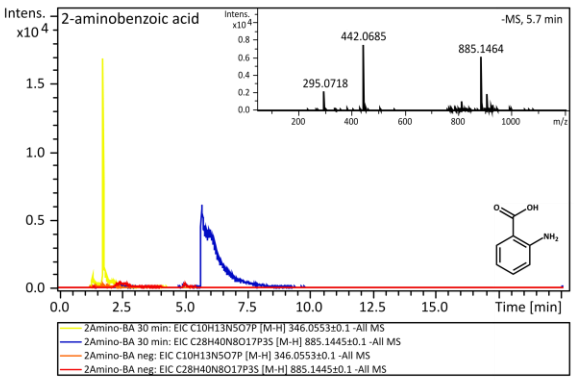

**Suppl. Fig. S9** Assays of Aa4HBCL (14 µg protein) with different benzoic acid derivatives measured by the indirect method with hexokinase and glucose-6-phosphate dehydrogenase. (a) Aa4HBCL incubated with 500 µM benzoic acid, monohydroxylated or dihydroxylated benzoic acids and 500 µM ATP and 500 µM CoA for 1 h at 40 °C before addition of reaction mixtures containing glucose, hexokinase, NADP and glucose-6-phosphate dehydrogenase. MeOH instead of substrate was used as a negative control. (b) Decreasing ATP concentration of Aa4HBCL assays incubated with 500 µM benzoic acid (BA), 3-hydroxybenzoic acid (3HBA) or 4-hydroxybenzoic acid (4HBA) and 500 µM ATP and 500 µM CoA after 0, 5, 15 and 30 min of incubation at 45 °C before addition of reaction mixtures containing glucose, hexokinase, NADP and glucose-6-phosphate dehydrogenase (mean ± SD, n = 3). A calibration curve of different ATP concentrations was used to calculate the remaining ATP concentration in the assays

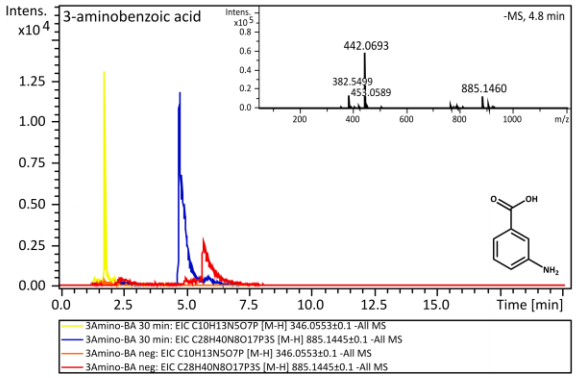

benzoic acid
-MS, 6.0 min
434.5647
870.1382
Time [min]
BA 30 min: EIC C10H13N5O7P [M-H]346.0553±0.1 -All MS
BA 30 min: EIC C28H39N7O17P3S [M-H]870.1336±0.1 -All MS
BA neg: EIC C10H13N5O7P [M-H]346.0553±0.1 -All MS
BA neg: EIC C28H39N7O17P3S [M-H]870.1336±0.1 -All MS

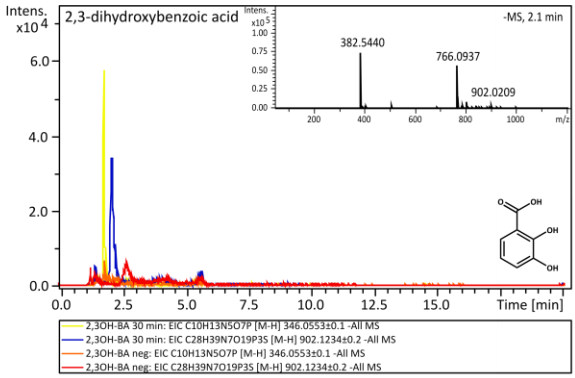

2-hydroxybenzoic acid
Time [min]
2HBA 30 min: EIC C10H13N5O7P [M-H]346.0553±0.1 -All MS
2HBA 30 min: EIC C28H39N7O18P3S [M-H]886.1285±0.1 -All MS
2HBA neg: EIC C10H13N5O7P [M-H]346.0553±0.1 -All MS
2HBA neg: EIC C28H39N7O18P3S [M-H]886.1285±0.1 -All MS

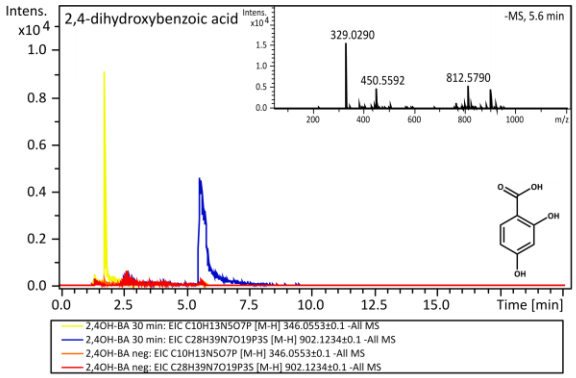

3-hydroxybenzoic acid
-MS, 5.4 min
442.5623
453.5519
886.1325
Time [min]
3HBA 30 min: EIC C10H13N5O7P [M-H] 346.0553±0.1 -All MS
3HBA 30 min: EIC C28H39N7O18P3S [M-H] 886.1285±0.1 -All MS
3HBA neg: EIC C10H13N5O7P [M-H] 346.0553±0.1 -All MS
3HBA neg: EIC C28H39N7O18P3S [M-H] 886.1285±0.1 -All MS

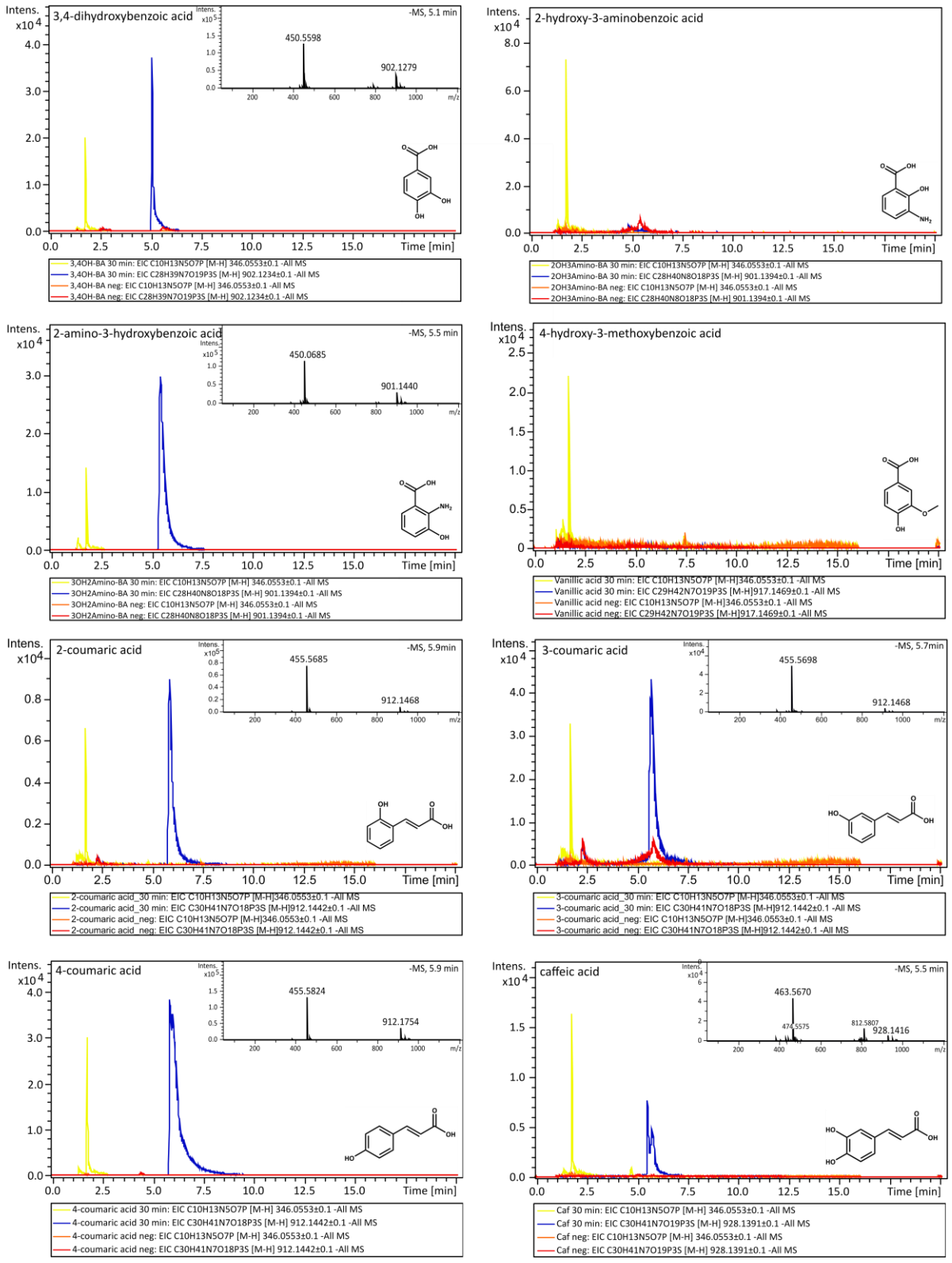

4-hydroxybenzoic acid
-MS, 5.4min
442.5631
886.1340
Time [min]
4HBA 30 min: EIC C10H13N5O7P [M-H] 346.0553±0.1 -All MS
4HBA 30 min: EIC C28H39N7O18P3S [M-H] 886.1285±0.1 -All MS
4HBA neg: EIC C10H13N5O7P [M-H] 346.0553±0.1 -All MS
4HBA neg: EIC C28H39N7O18P3S [M-H] 886.1285±0.1 -All MS


2-aminobenzoic acid
-MS, 5.7 min
442.0685
885.1464
295.0718
Time [min]
2Amino-BA 30 min: EIC C10H13N5O7P [M-H] 346.0553±0.1 -All MS
2Amino-BA 30 min: EIC C28H40N8O17P3S [M-H] 885.1445±0.1 -All MS
2Amino-BA neg: EIC C10H13N5O7P [M-H] 346.0553±0.1 -All MS
2Amino-BA neg: EIC C28H40N8O17P3S [M-H] 885.1445±0.1 -All MS


3-aminobenzoic acid
-MS, 4.8 min
442.0693
382.5409
453.0589
885.1460
Time [min]
3Amino-BA 30 min: EIC C10H13N5O7P [M-H] 346.0553±0.1 -All MS
3Amino-BA 30 min: EIC C28H40N8O17P3S [M-H] 885.1445±0.1 -All MS
3Amino-BA neg: EIC C10H13N5O7P [M-H] 346.0553±0.1 -All MS
3Amino-BA neg: EIC C28H40N8O17P3S [M-H] 885.1445±0.1 -All MS

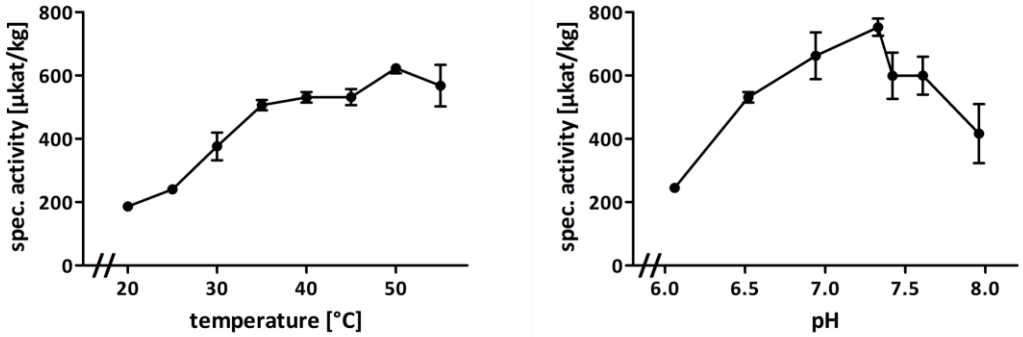

2,3-dihydroxybenzoic acid
-MS, 2.1 min
382.5440
766.0937
902.0209
Time [min]
2,3OH-BA 30 min: EIC C10H13N5O7P [M-H] 346.0553±0.1 -All MS
2,3OH-BA 30 min: EIC C28H39N7O19P3S [M-H] 902.1234±0.2 -All MS
2,3OH-BA neg: EIC C10H13N5O7P [M-H] 346.0553±0.1 -All MS
2,3OH-BA neg: EIC C28H39N7O19P3S [M-H] 902.1234±0.2 -All MS


2,4-dihydroxybenzoic acid
-MS, 5.6 min
329.0290
450.5592
812.5790
Time [min]
2,4OH-BA 30 min: EIC C10H13N5O7P [M-H] 346.0553±0.1 -All MS
2,4OH-BA 30 min: EIC C28H39N7O19P3S [M-H] 902.1234±0.1 -All MS
2,4OH-BA neg: EIC C10H13N5O7P [M-H] 346.0553±0.1 -All MS
2,4OH-BA neg: EIC C28H39N7O19P3S [M-H] 902.1234±0.1 -All MS

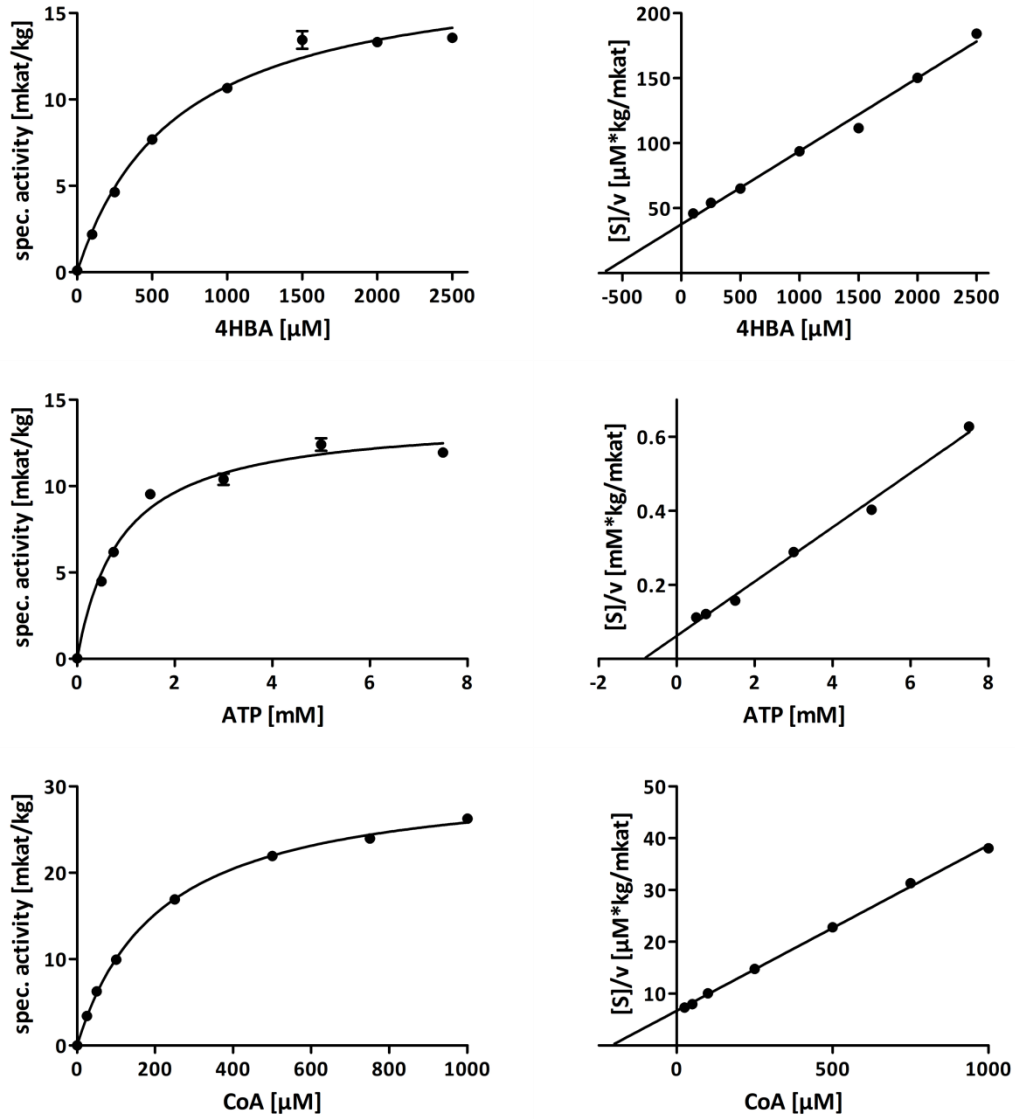



**Suppl. Fig. S10** LC-MS analysis of reaction products of Aa4HBCL with different (hydroxy)benzoic and (hydroxy)cinnamic acids. Purified Aa4HBCL (28 µg protein) was incubated with 500 µM substrate, 1.25 mM ATP and 1 mM CoA for 30 min at 40 °C. Heat-denatured protein (10 min 95 °C) was used as a negative control. The chromatograms show the EIC of the expected products AMP (m/z 346.0553 ± 0.1) in yellow for Aa4HBCL and

orange for the negative control. The corresponding CoA-ester is displayed in blue for Aa4HBCL or red for the negative control. The exact mass of the resulting CoA-ester is shown in each chromatogram. For all produced CoA-esters we observed the [M-H] pseudo molecular ion as well as the doubly charged molecular ion [M/2]-H




**Suppl. Fig. S11** Temperature (left) and pH-optimum (right) of Aa4HBCL (mean ± SD, n = 3)




**Suppl. Fig. S12** Dependence of Aa4HBCL on 4-hydroxybenzoic acid (4HBA), ATP and CoA for the determination of $K_m$-values (mean ± SD, n = 9). Michaelis-Menten diagrams are displayed on the left and Hanes-Wolf diagrams on the right. 4HBA was incubated with 4 mM ATP and 750 µM CoA. Determination of $K_m$-values for ATP were measured with 2.5 mM 4HBA and 750 µM CoA. Different CoA concentrations were incubated with 2.5 mM 4HBA and 5 mM ATP