Directed Multistep Biocatalysis for the Synthesis of the Polyketide Oxytetracycline in Permeabilized Cells of *Escherichia coli*

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Supporting information

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1. High resolution mass analysis of dihydro-oxytetracycline

Since the dihydro-oxytetracycline is unknown in the literature, additional characterization was carried out. Full structural characterization (NMR, IR, MS) would require substantial amounts of the purified compounds. In the 25 mL setup we were able to synthesize only small amounts (0.9 µg/L OTC, H2OTC and H4OTC combined). Therefore, we generated high resolution MALDI-FT-ICR-MS data.

For high resolution MALDI-FTICR-MS analysis, external linear calibration was performed using a commercial standard for oxytetracycline (Sigma-Aldrich, >99% purity). According to the theoretical mass listed in Table S1, three monoisotopic peaks were used to calibrate the FTICR signal, yielding high resolution m/z data.

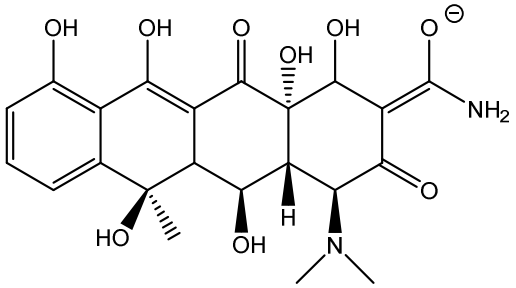
Table S1: Ions used for calibration of MS-data.

Ion	m/z	z
[M-H20]	441.129793	-1
[M-NH3]	442.113809	-1
Oxytetracycline	459.140358	-1

High-resolution m/z values measured from culture samples (Table S2) reveal that in most cases the experimentally obtained mass was very close to the expected value H_2OTC of 461.15655 (Table S3). Only the value at 21 h differed more from the theoretically expected mass (24.67 ppm). This may be due to the much lower measured intensity (Table S2). The average experimental m/z is 461.15713 ± 0.00019 with an average deviation from the theoretical mass of 1.25±0.41 ppm (not considering the value at 21 h).

Table S2: MALDI-FTICR-MS high resolution data for peaks at 461,15 detected in samples of OTC synthesis.

Sample	m/z	Intensity	Resonance frequency [Hz]
5 min	461.15698	2516925	73607
1 h	461.15742	6851083	73575
15 h	461.15717	1763063	78344
21 h	461.16793	470832	64779
38 h	461.15695	4240308	73296



Scheme S1: Proposed structure of the dihydro-oxytetracycline single negative charged ion. Chemical formula: $C_{22}H_{25}N_2O_9^-$, monoisotopic mass: 461.15655.

Table S3: Mass differences in ppm of peaks at m/z 461.1 compared to the theoretical monoisotopic mass of H_2OTC

Sample	m/z		Δ ppm
	experimental	theoretical	
5min	461.15698	461.15655	0.93
1h	461.15742	461.15655	1.88
15h	461.15717	461.15655	1.34
21h	461.16793	461.15655	24.67
38h	461.15695	461.15655	0.86

According to FTICR-MS data, there was no significant amount of H_4OTC present in the samples from 5 minutes to 15 hours and 38 hours. Only the 21 h sample did show a signal at 463.1667 that has however a mass difference of 11.87 ppm to the theoretical mass of 463.17220. Therefore, we cannot conclude that H_4OTC was present in that sample.

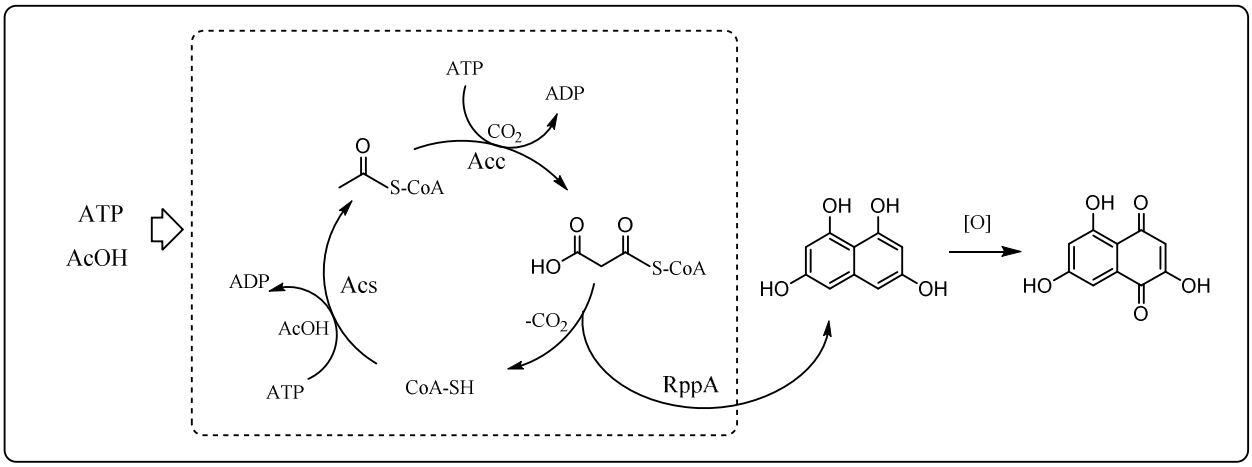
2. Kinetic data of the NADPH supply module

Table S4: NADPH generation activity of cells in Tris-HCl buffer (0.1 M) containing 4 g/L sodium phosphonate and 5 mM sodium nitrate coupled to a nitrate reductase assay.

	Activity of NADPH generation according to biocatalyst and substrate [$\frac{nmol_{NADPH}}{g_{cells}\, x\, h}$]		
	NADPH	NADP+	none
E. coli BAPI pRSF-ptdH (permeabilized)	-0,65	12,52	15,10
E. coli BWZ 3 pRSF-ptdH (permeabilized)	47,29	51,68	71,87
E. coli BWZ 3 (permeabilized)	-5,03	9,42	10,06
E coli BWZ 3 (native)	1,94	2,52	1,29

3. Synthesis of flaviolin in permeabilized cells

For the synthesis of flaviolin, the polyketide type III synthase rppA was expressed in *E. coli* BWZ3. According to the biosynthesis in Scheme S2 one molecule of flaviolin requires 10 molecules of ATP.



Scheme S2: Synthesis of Flaviolin by permeabilized cells of *E.coli* BWZ3 pET-*rppA*/pACYC-*acs* in reaction buffer containing 30 mM ATP, 40 mM acetate, 50 µM coenzyme A and 0,1 M NH_4HCO_3. Starting from acetate and ATP, acetyl-CoA is generated and carboxylated yielding malonyl-CoA. Tetrahydroxy-naphthalene is generated by a polyketide type III synthase (RppA) in 5 consecutive chain elongation steps using malonyl-CoA. Further intramolecular cyclization and non-enzymatic oxidation yields flaviolin, which can be detected in a photometric assay at 520 nm.

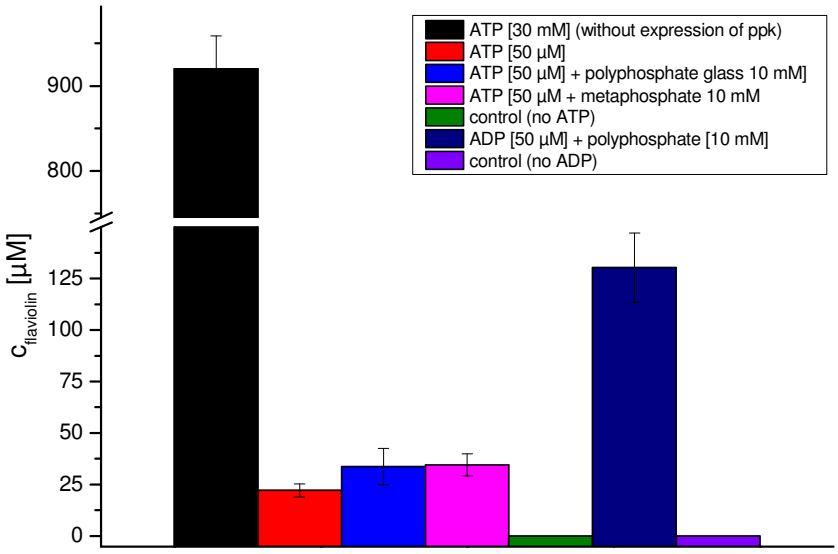


Figure S1: Concentration of flaviolin observed in reactions with different types of ATP supply (compare Table 2 in the paper). Bar 1 shows the concentration of Flaviolin generated by direct supply of 30 mM ATP. Bars 2-4 show concentrations of flaviolin generated with regeneration of supplied ATP (50 µM) via polyphosphate kinase, bar 5 is the control. Bar 6 shows the generation of flaviolin with ADP supplied in the presence of a polyphosphate kinase and adenylate kinase ATP regeneration system, while bar 7 is the control.

4. Theoretical consumption of substrates and cofactors for OTC synthesis

In Table S5 the theoretically required amounts of substrates and cofactors used for the synthesis of 1.95 nM OTC are listed.

Table S5: Concentration of substances used/generated during synthesis of OTC based on the yield of OTC and H_2OTC and the stoichiometry of OTC biosynthesis

Substance	Concentration of OTC and distribution of substrates [nM]
OTC/H_2OTC	1.95
ATP	44.96
NADPH	3.91
SAM (methionine)	5.86
acetate	19.55