The versatility of non-heme diiron monooxygenase PmlABCDEF: a single biocatalyst for a plethora of oxygenation reactions

Supporting information

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General Remarks

NMR spectroscopy was performed on a Bruker Ascend 400. NMR spectra were recorded at ambient temperature using DMSO-d_6 and $CDCl_3$ as solvents, with proton, and carbon resonances at 400 and 101 Mhz, respectively. All NMR data are reported in ppm relative to the solvent signal as the internal standard. The multiplicities are stated as follows: s = singlet, br s = broad singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet. Proton spectra were readjusted for presentation by removing solvent peaks.

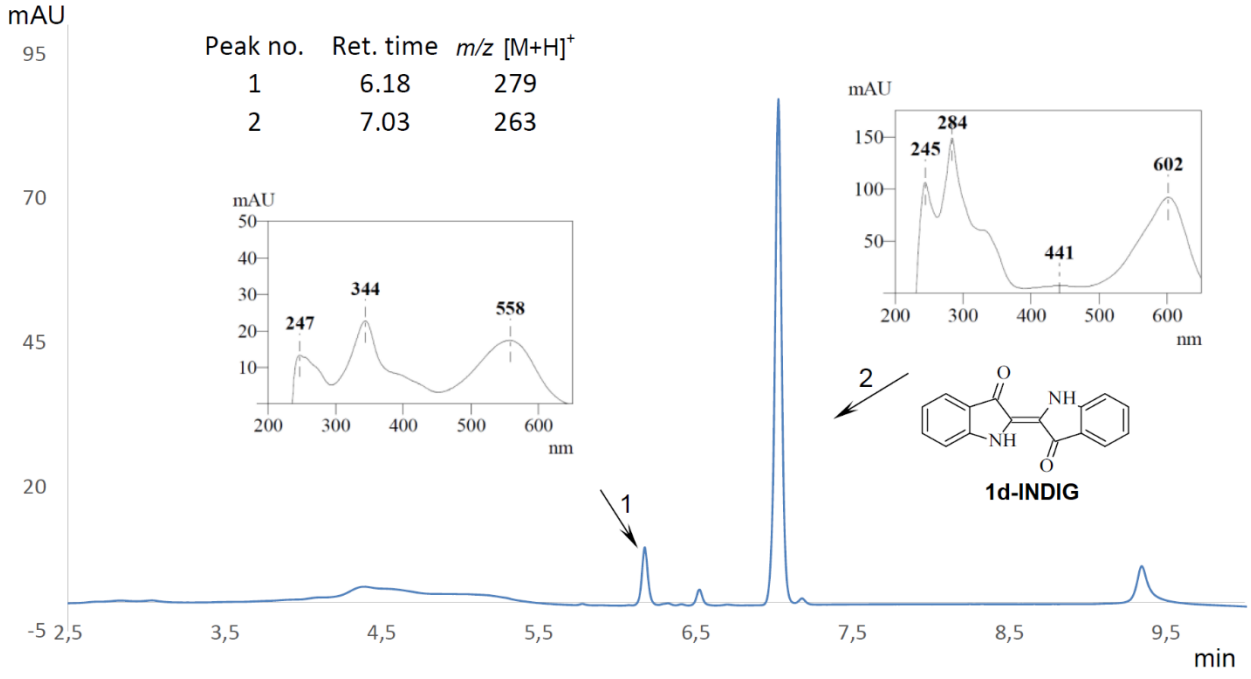
All the HPLC-MS analyses were performed with a high-performance liquid chromatography system (Shimadzu, Japan) and a mass spectrometer (LCMS-2020, Shimadzu, Japan) equipped with an ESI source. The chromatographic separation was conducted using a YMC Pack Pro C18 column, 3 × 150 mm (YMC, Japan) at 40 °C and a mobile phase that consisted of 0.1% formic acid water solution (solvent A), and acetonitrile (solvent B) delivered in the 5 → 95% gradient elution mode. Mass scans were measured from m/z 50 up to m/z 700, at 350 °C interface temperature, 250 °C DL temperature, ±4,500 V interface voltage, neutral DL/Qarray, using N_2 as nebulizing and drying gas. Mass spectrometry data were acquired in both the positive and negative ionization modes.

GC-MS analysis was performed with a Shimadzu Nexis GC_2030 connected to a GCMS-QP2020 NX (Shimadzu, Japan) equipped with a FS-Supreme-5ms column (Ziemer, Germany).

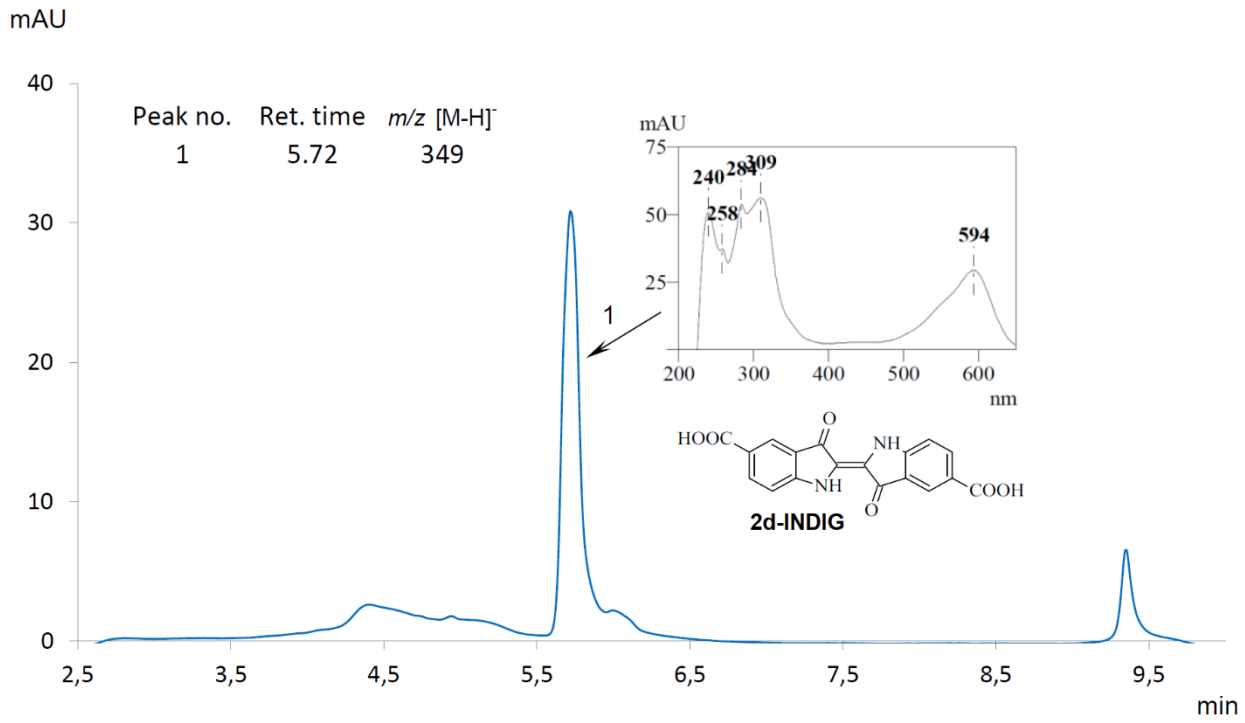
Compounds subjected to NMR analysis were synthesized and purified according to the general biocatalysis procedures which were described in the main text.

HPLC-MS chromatograms of indigoid compounds

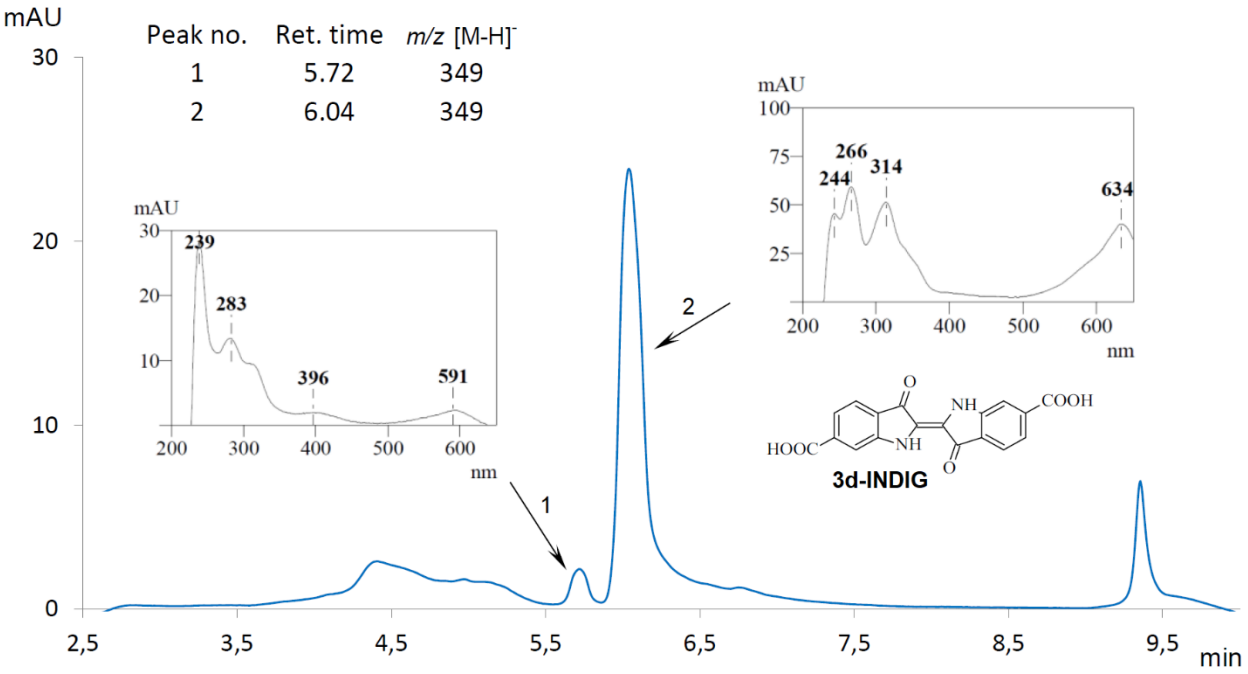
The sample (0.5 mL) of the whole-cell biocatalysis reaction was transferred to a 1.5 ml tube and mixed with an equal part of chloroform. The mixture was intensively shaken followed by centrifugation (12000 *g* for 5 min), 0.1 ml of organic extract was combined with 0.4 mL of acetonitrile, and was analysed with HPLC-MS. Chromatograms were generated using a wavelength of 600 nm. Products featuring distinctive UV-Vis spectra and the proper m/z $[M+H]^+$ or m/z $[M-H]^-$ value were presented. Known compounds were designated by labelling the peak in a chromatogram with appropriate structural formula.



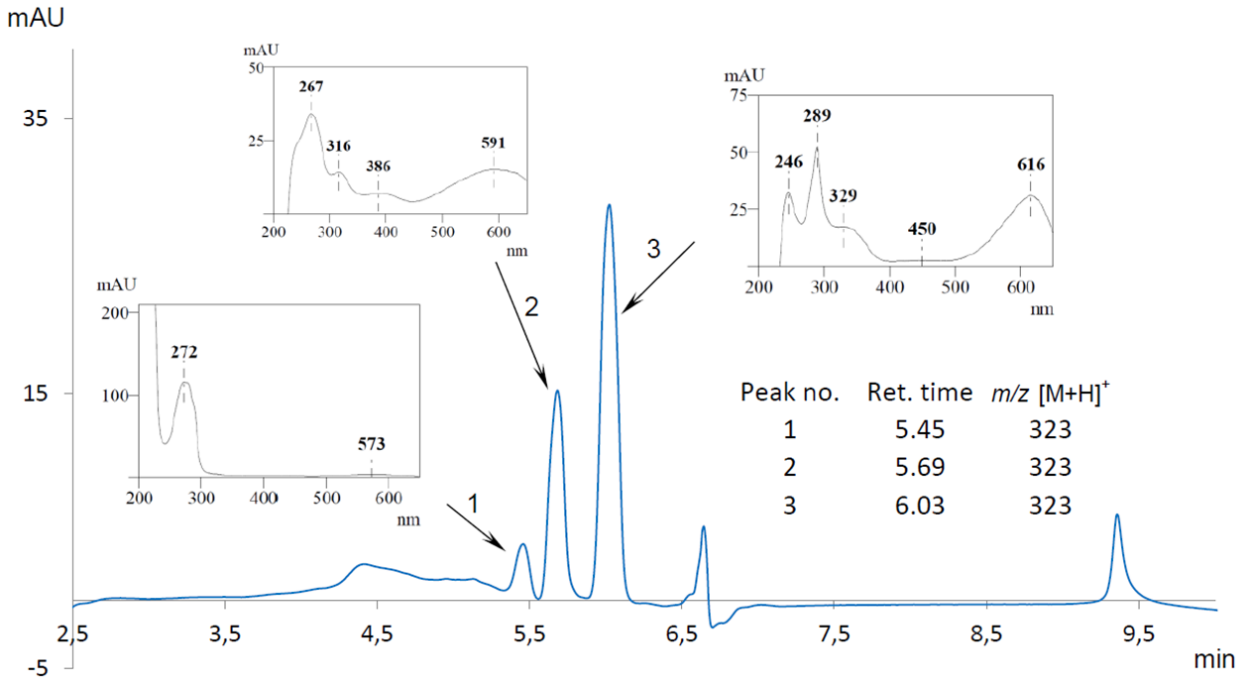
Products of indole (**1d**) conversion employing wild type PmlABCDEF.



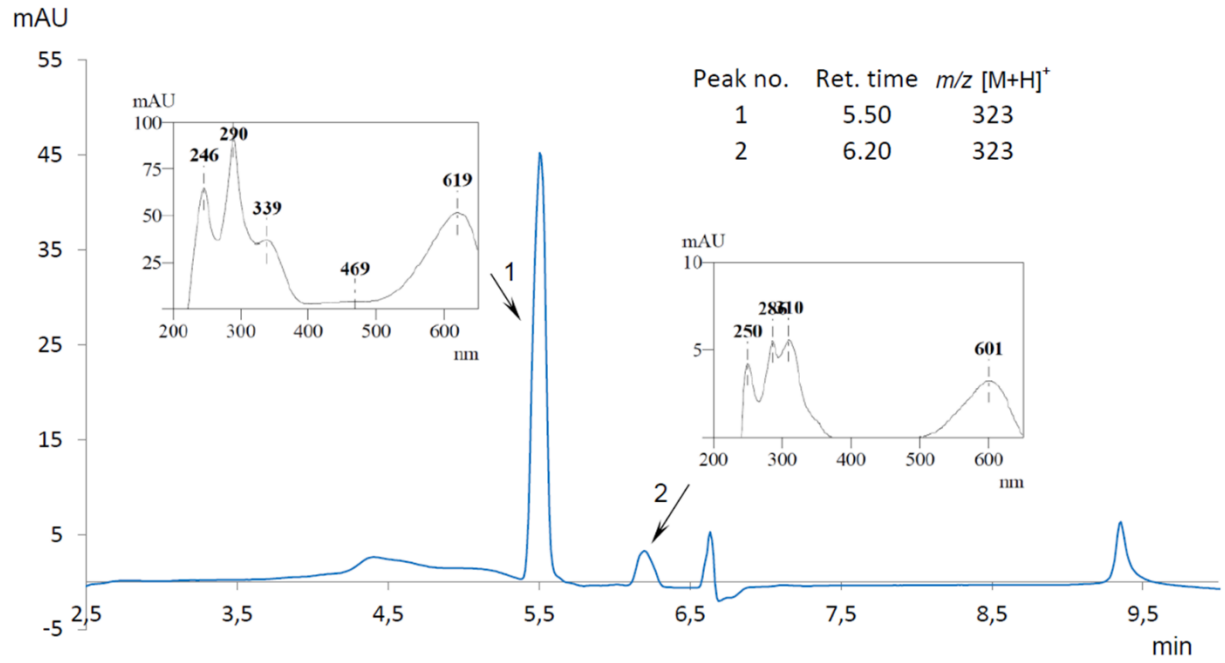
Products of indole-5-carboxylic acid (**2d**) conversion employing PmlD G109Q mutant of PmlABCDEF.



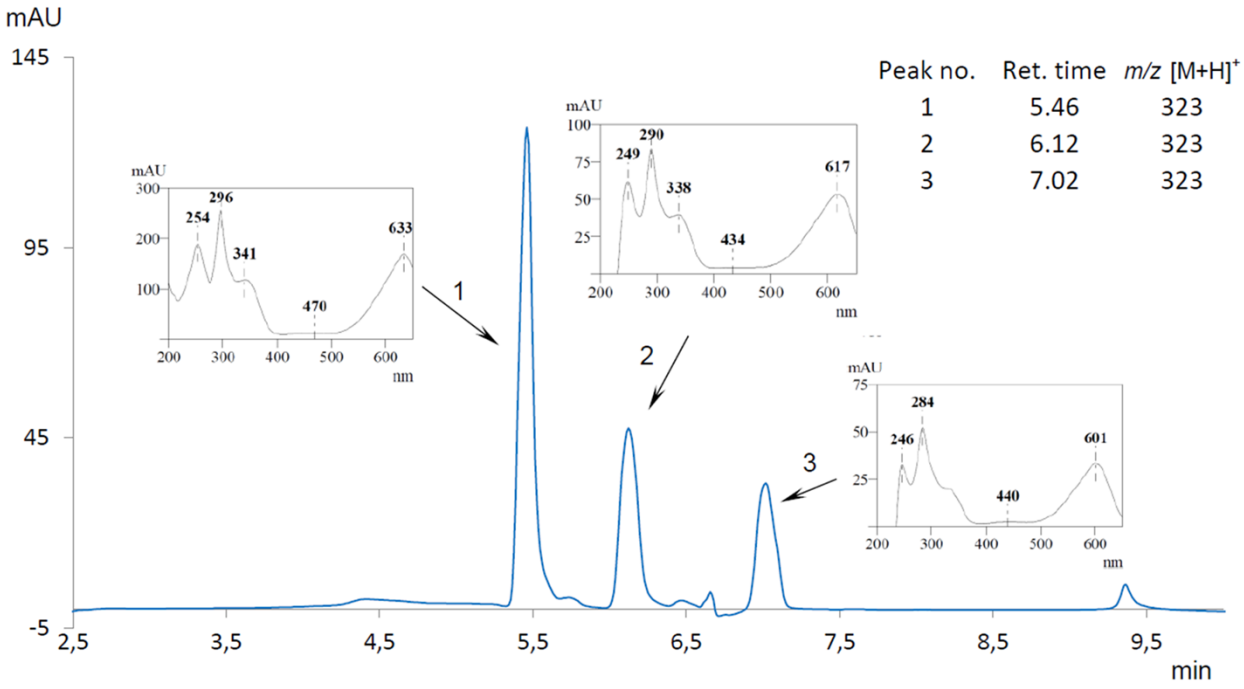
Products of indole-6-carboxylic acid (**3d**) conversion employing PmlD G109Q mutant of PmlABCDEF.



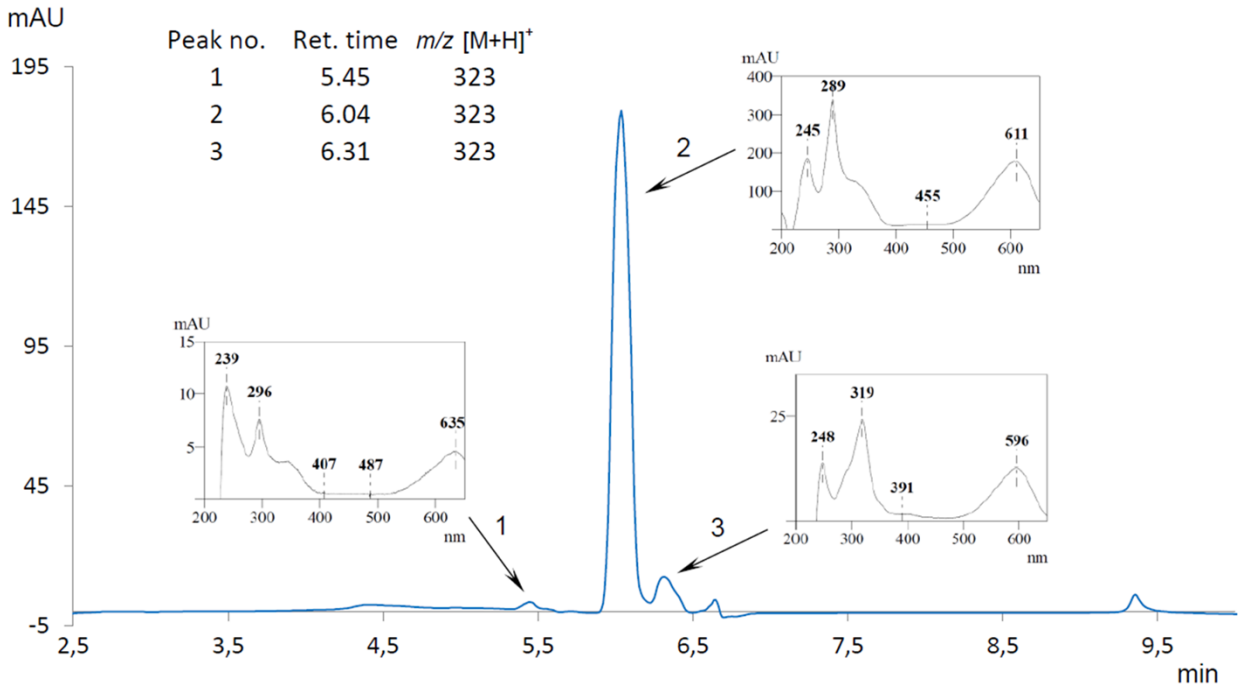
Products of indole-4-methanol (**4d**) conversion employing wild type PmlABCDEF.



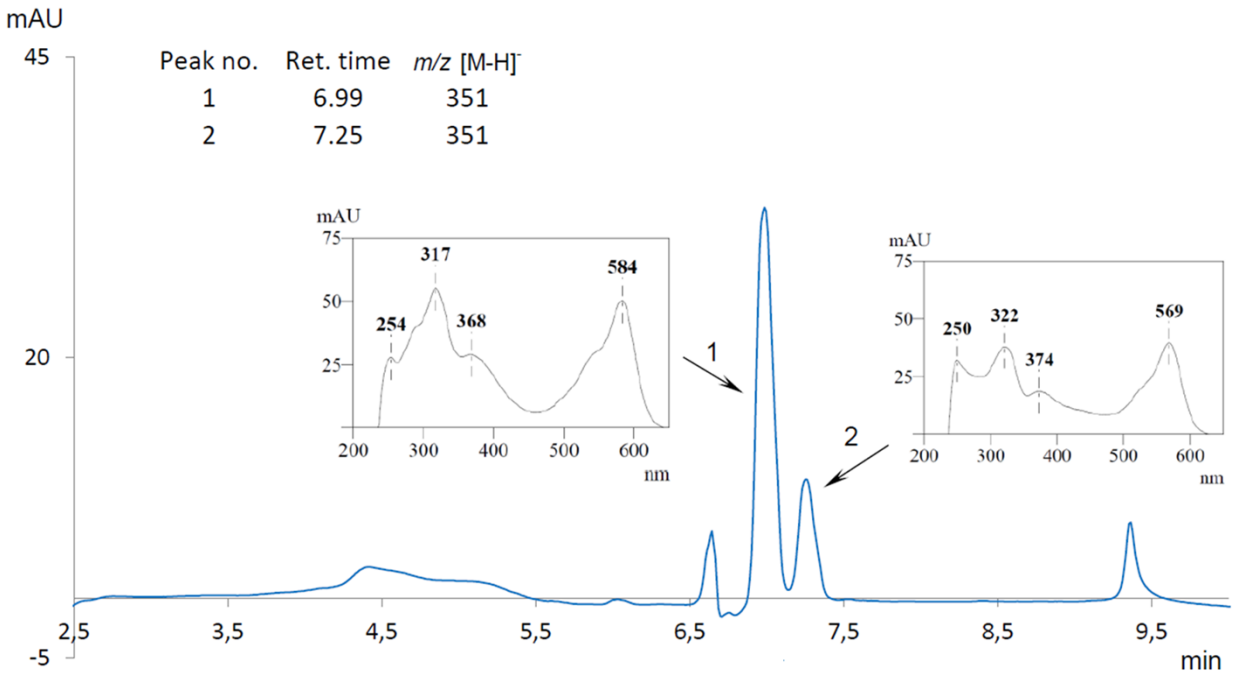
Products of indole-5-methanol (**5d**) conversion employing wild type PmlABCDEF.



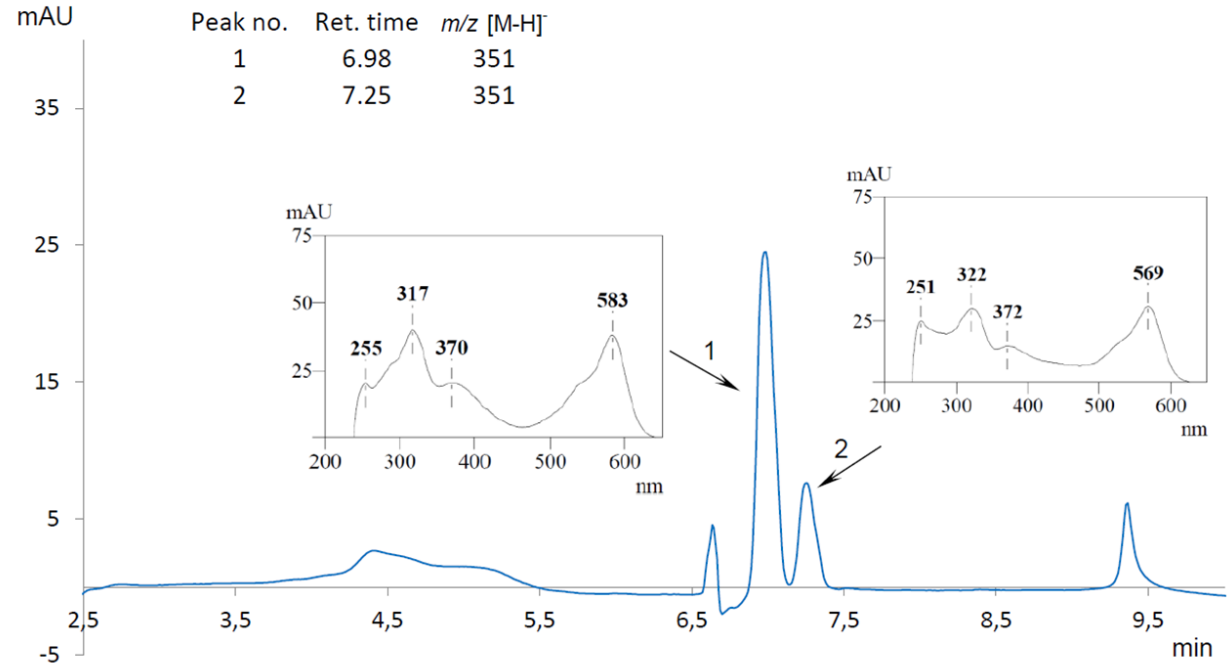
Products of indole-6-methanol (**6d**) conversion employing wild type PmlABCDEF.



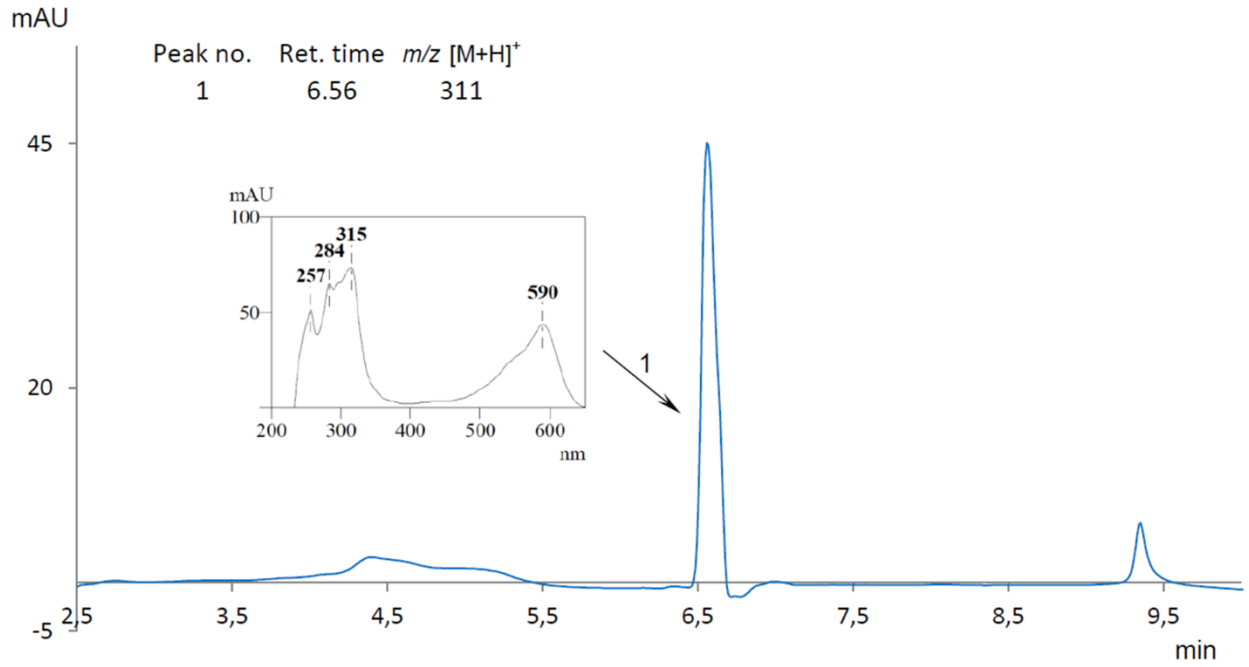
Products of indole-7-methanol (**7d**) conversion employing wild type PmlABCDEF.



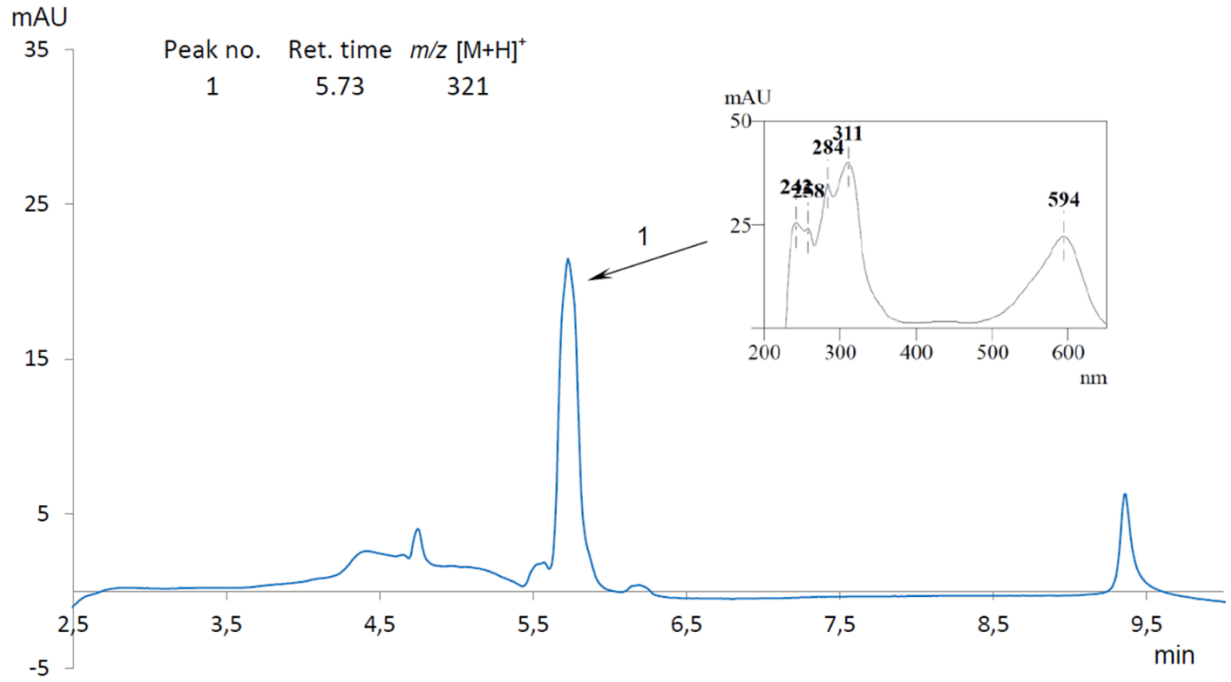
Products of 5-nitroindole (**8d**) conversion employing wild type PmlABCDEF.



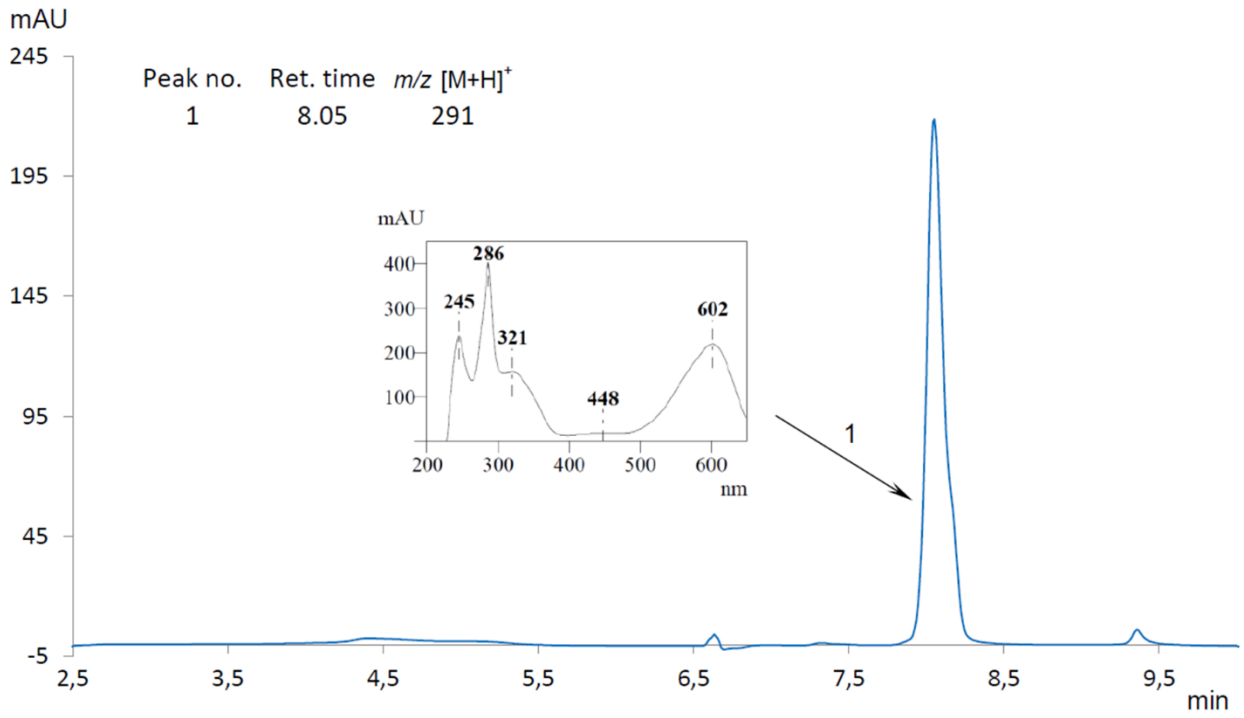
Products of 7-nitroindole (**9d**) conversion employing wild type PmlABCDEF.



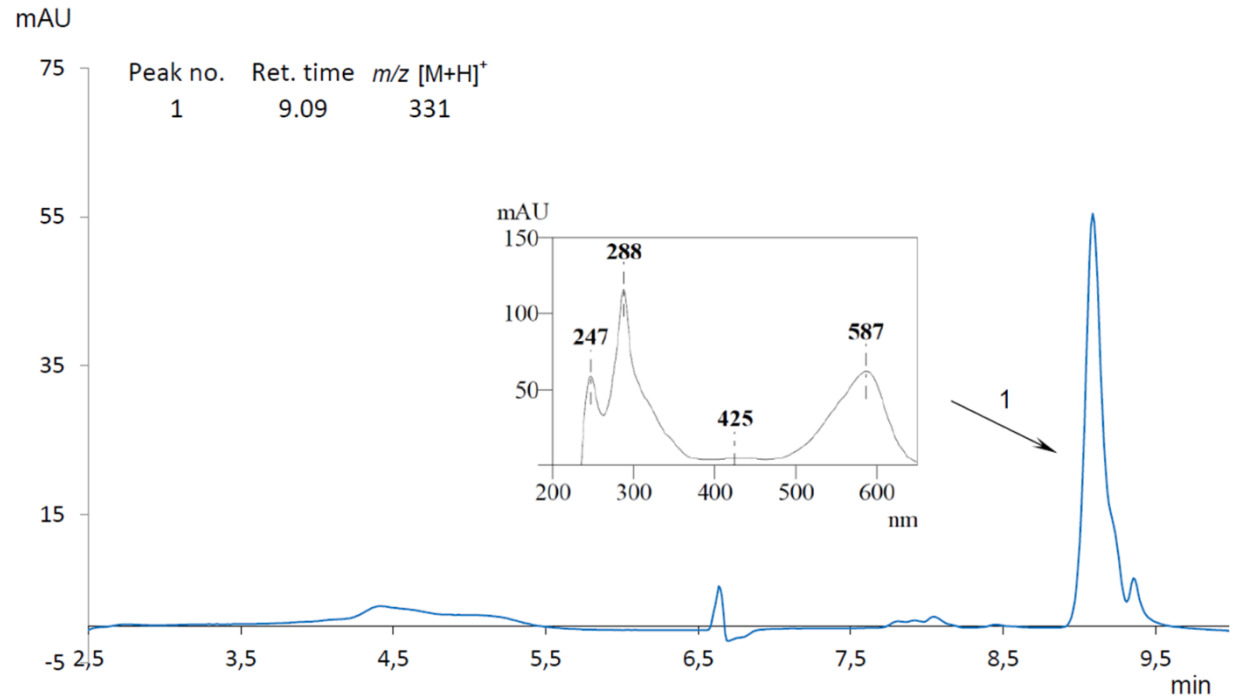
Products of indole-5-carbonitrile (**10d**) conversion employing wild type PmlABCDEF.



Products of indole-5-methanamine (**11d**) conversion employing wild type PmlABCDEF.



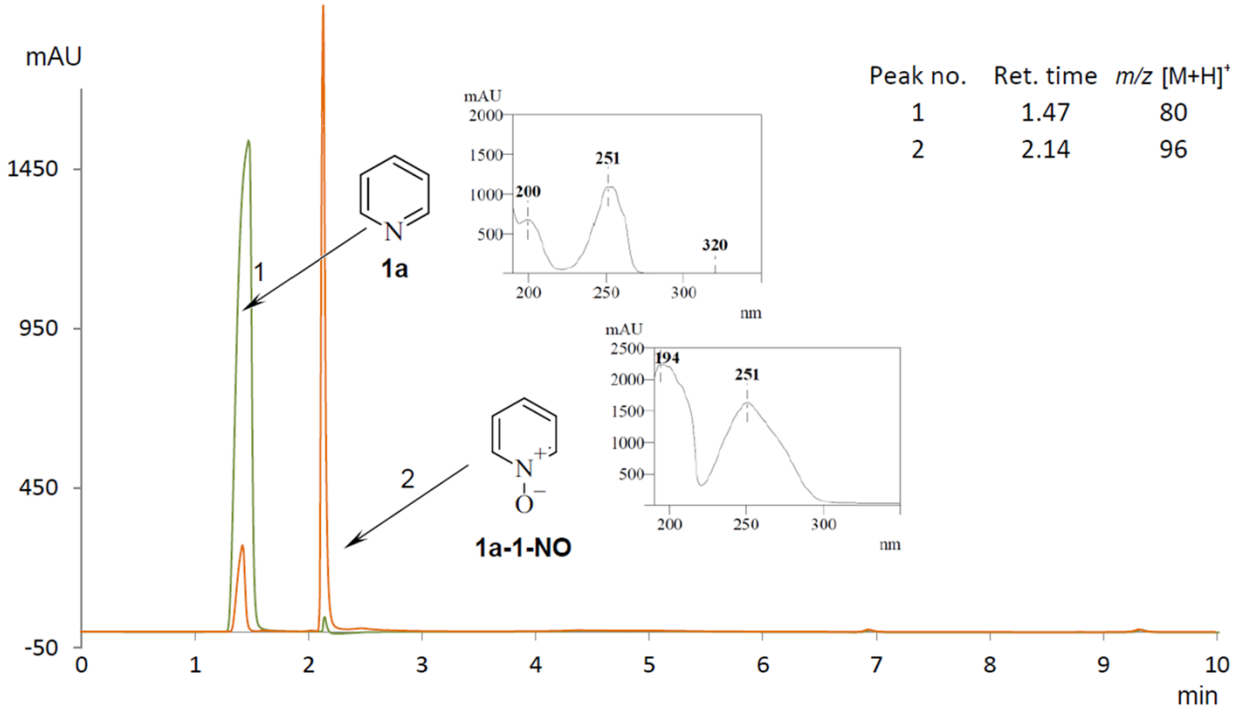
Products of 7-methylindole (**12d**) conversion employing wild type PmlABCDEF.



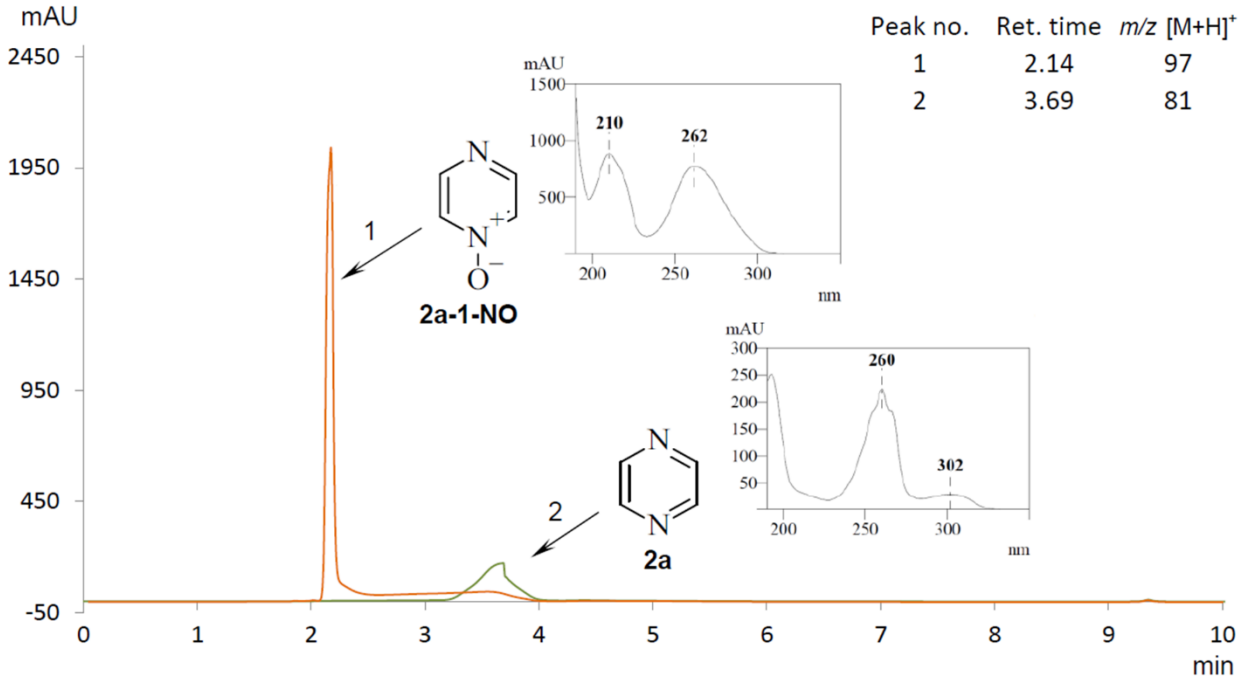
Products of 7-chloroindole (**13d**) conversion employing wild type PmlABCDEF.

HPLC-MS chromatograms of *N*-oxides, catechols, and sulfoxides

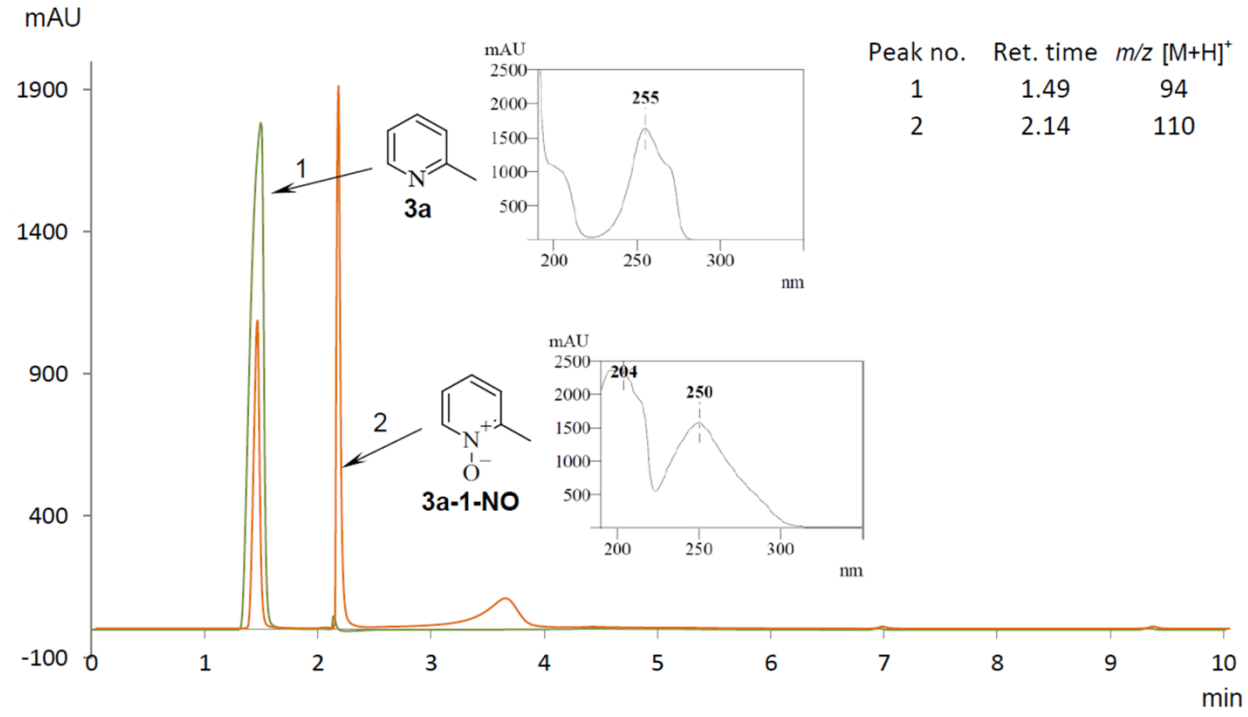
The sample (0.5 mL) of the whole-cell biocatalysis reaction was transferred to a 1.5 mL tube and mixed with an equal part of acetonitrile. The mixture was intensively vortexed followed by centrifugation (12000 *g* for 5 min), an injection of supernatant was analysed with HPLC-MS. Stacked chromatograms were generated using wavelengths of 254 nm (*N*-oxides, sulfoxides) and 270 nm (catechols). Reactions with *P. putida* KT2440 whole cells producing Pml monooxygenase are indicated by the orange line, control reactions (substrate incubated with *P. putida* KT2440 cells without *pml* gene) are displayed in the green line. Blue and brown lines indicate the appropriate standard compound.



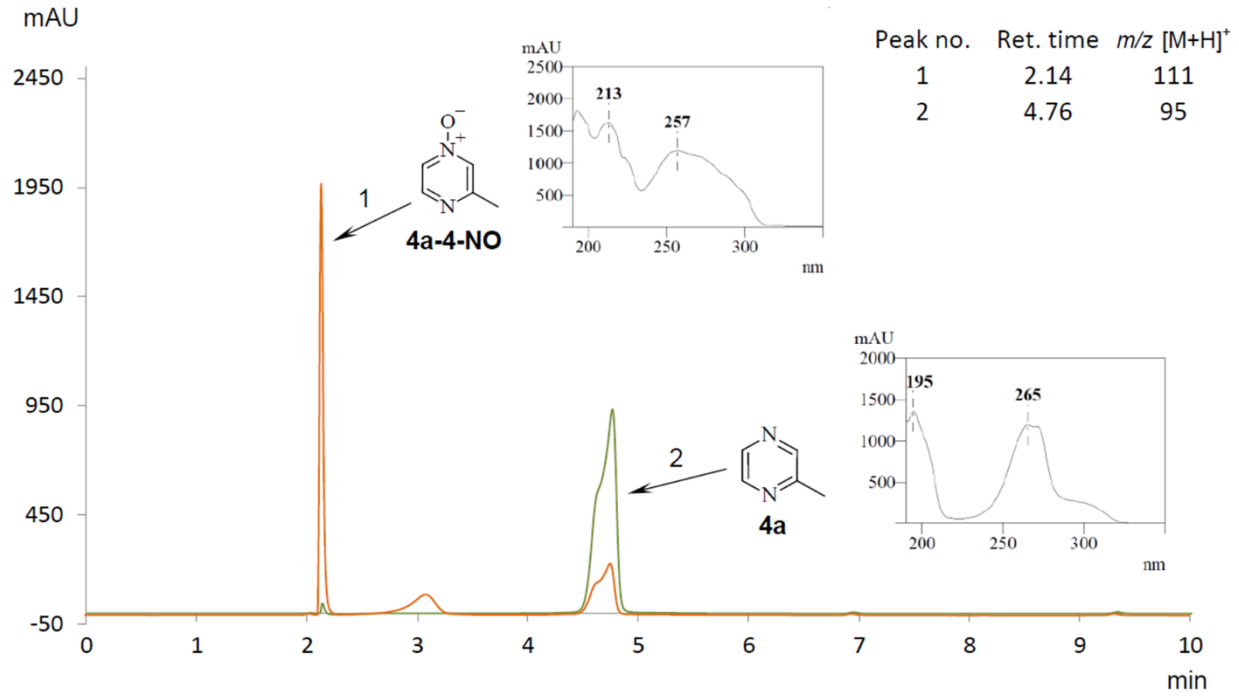
HPLC-MS analysis of pyridine (**1a**) conversion employing wild type PmlABCDEF.



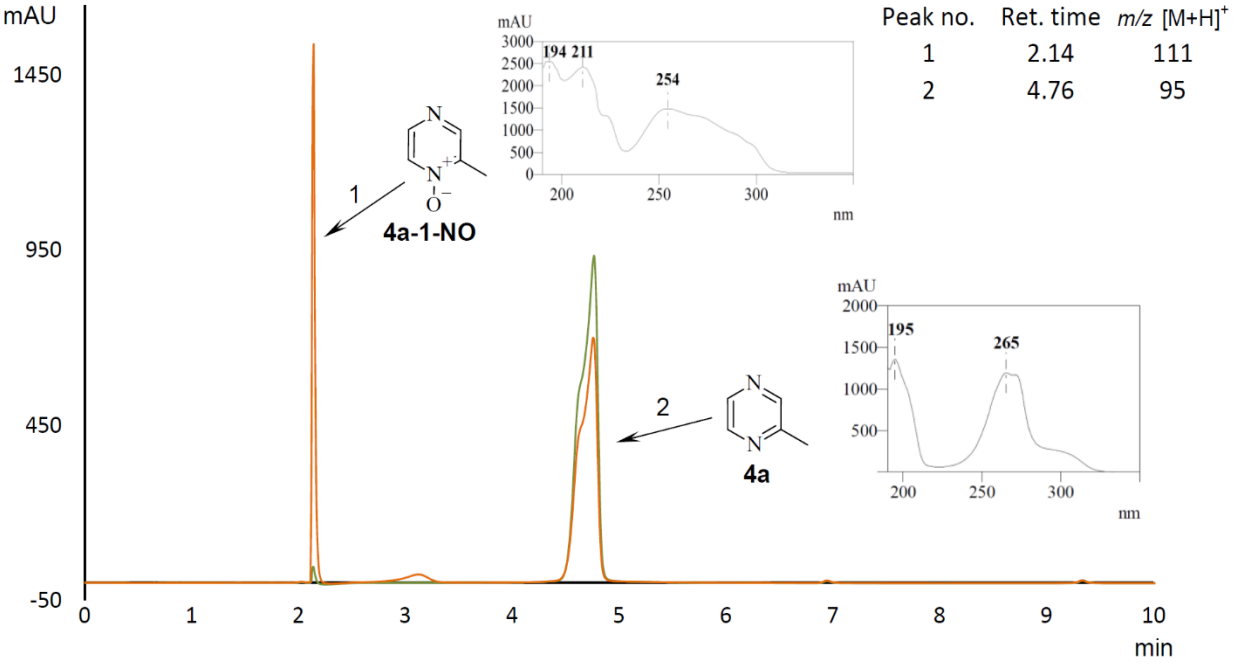
HPLC-MS analysis of pyrazine (**2a**) conversion employing wild type PmlABCDEF.



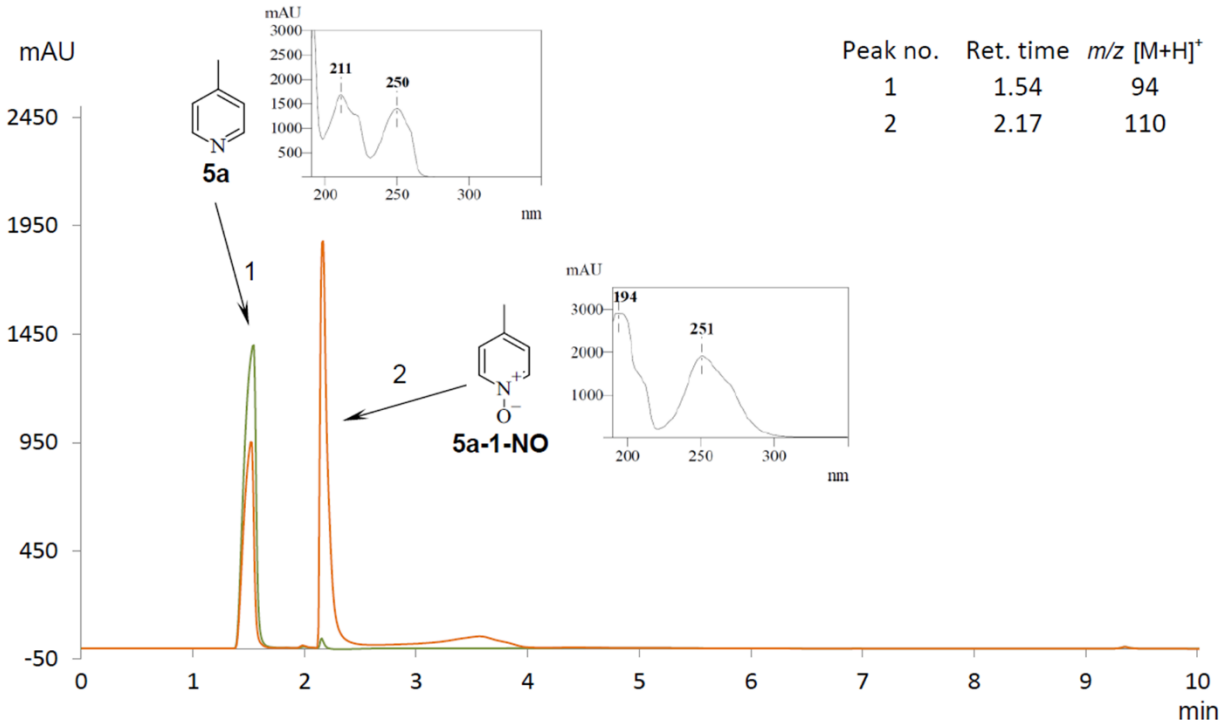
HPLC-MS analysis of 2-methylpyridine (**3a**) conversion employing wild type PmlABCDEF.



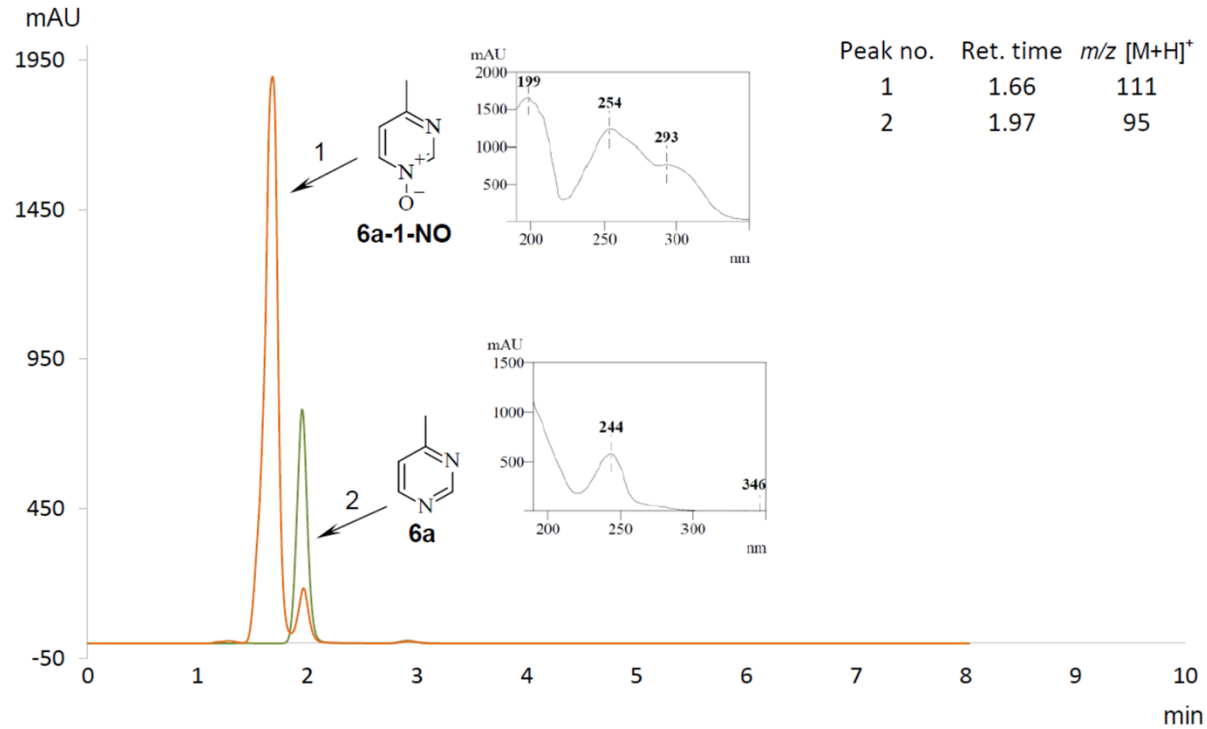
HPLC-MS analysis of 2-methylpyrazine (**4a**) conversion employing wild type PmlABCDEF.



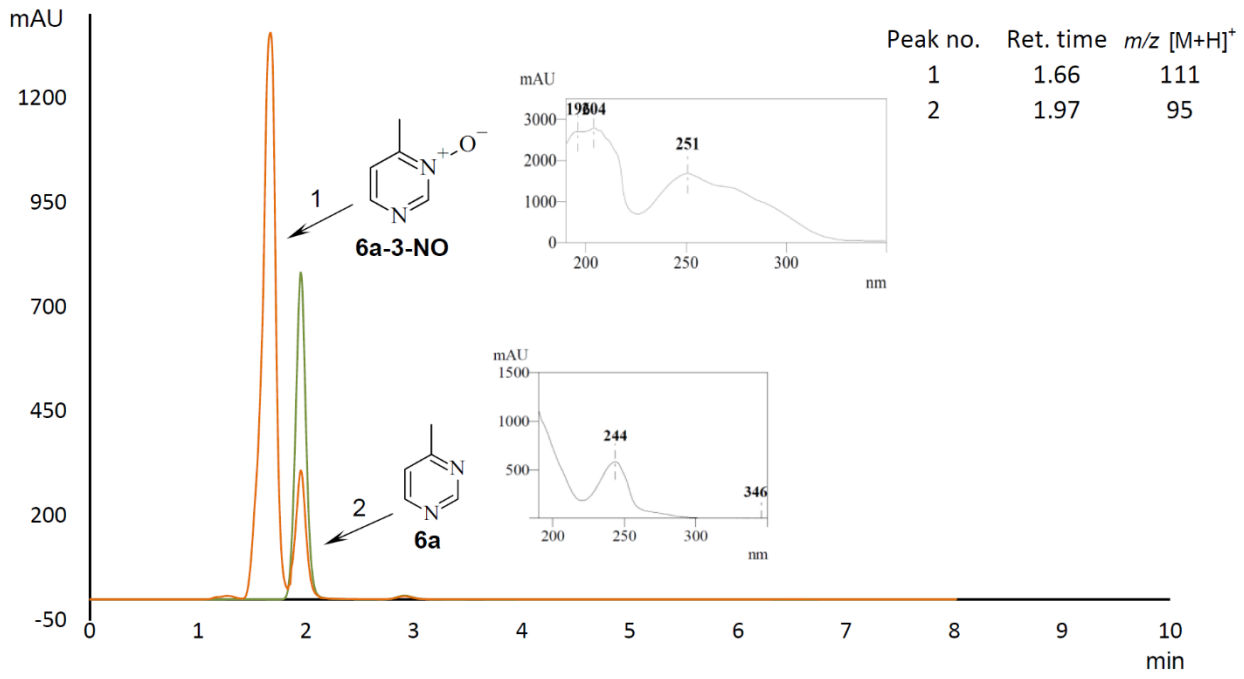
HPLC-MS analysis of 2-methylpyrazine (**4a**) conversion employing PmlD A113G mutant of PmlABCDEF.



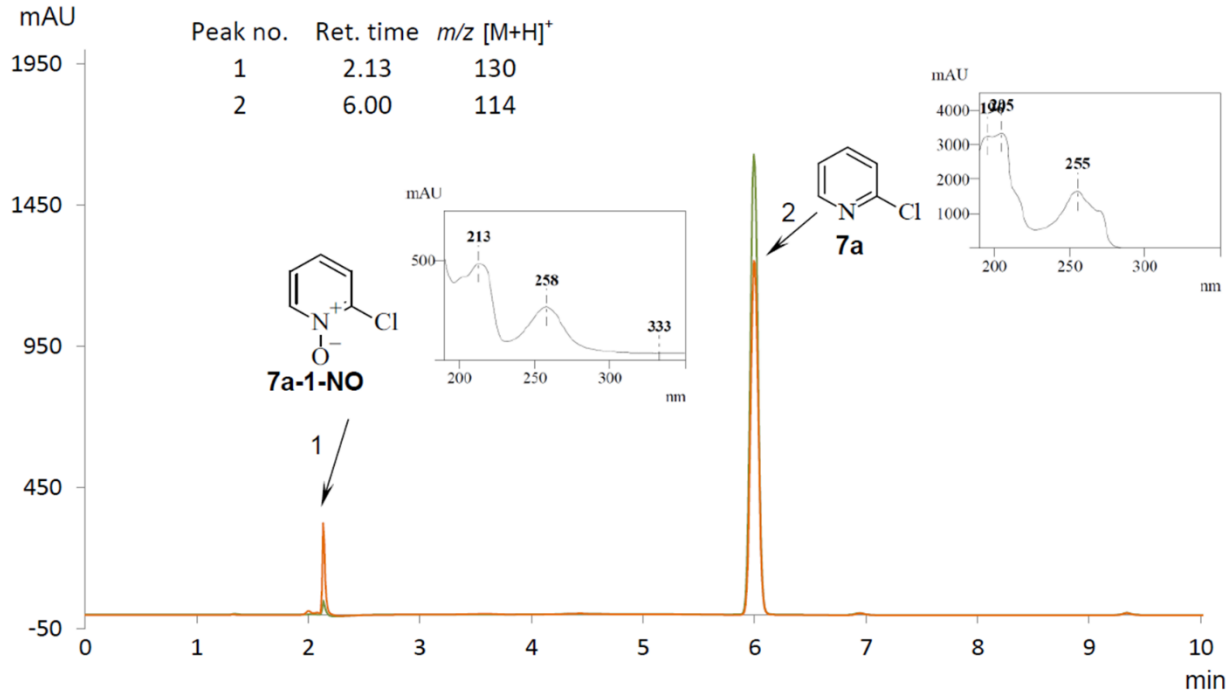
HPLC-MS analysis of 4-methylpyridine (**5a**) conversion employing wild type PmlABCDEF.



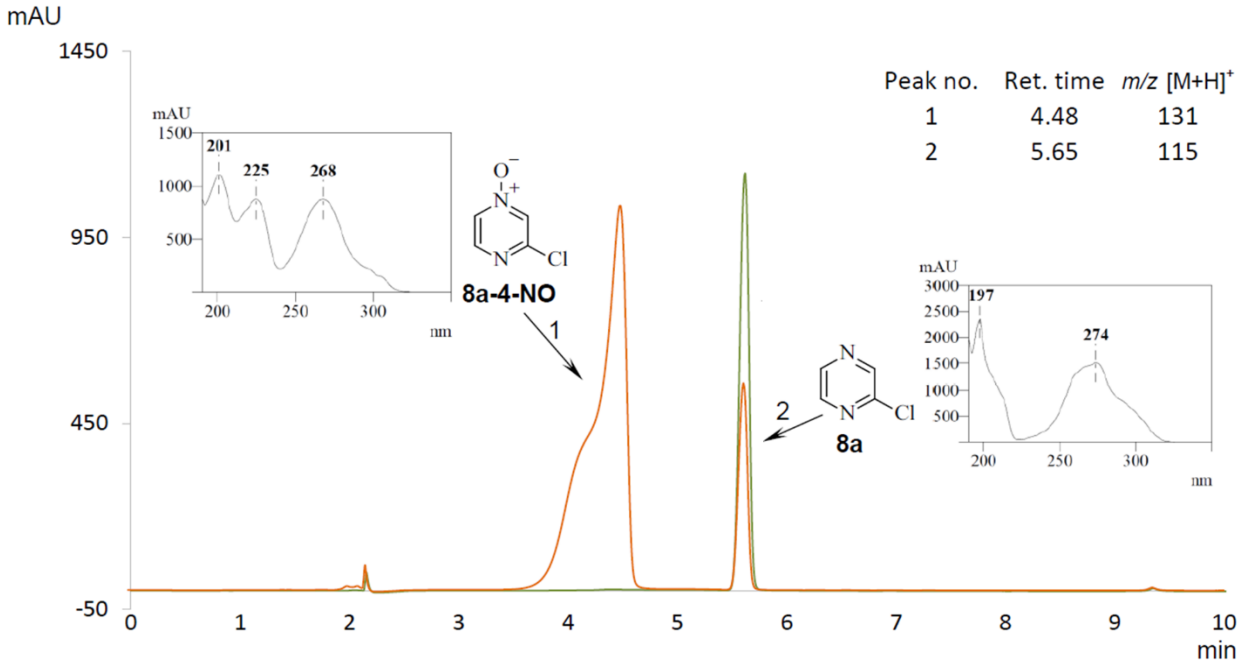
HPLC-MS analysis of 4-methylpyrimidine (**6a**) conversion employing wild type PmlABCDEF.



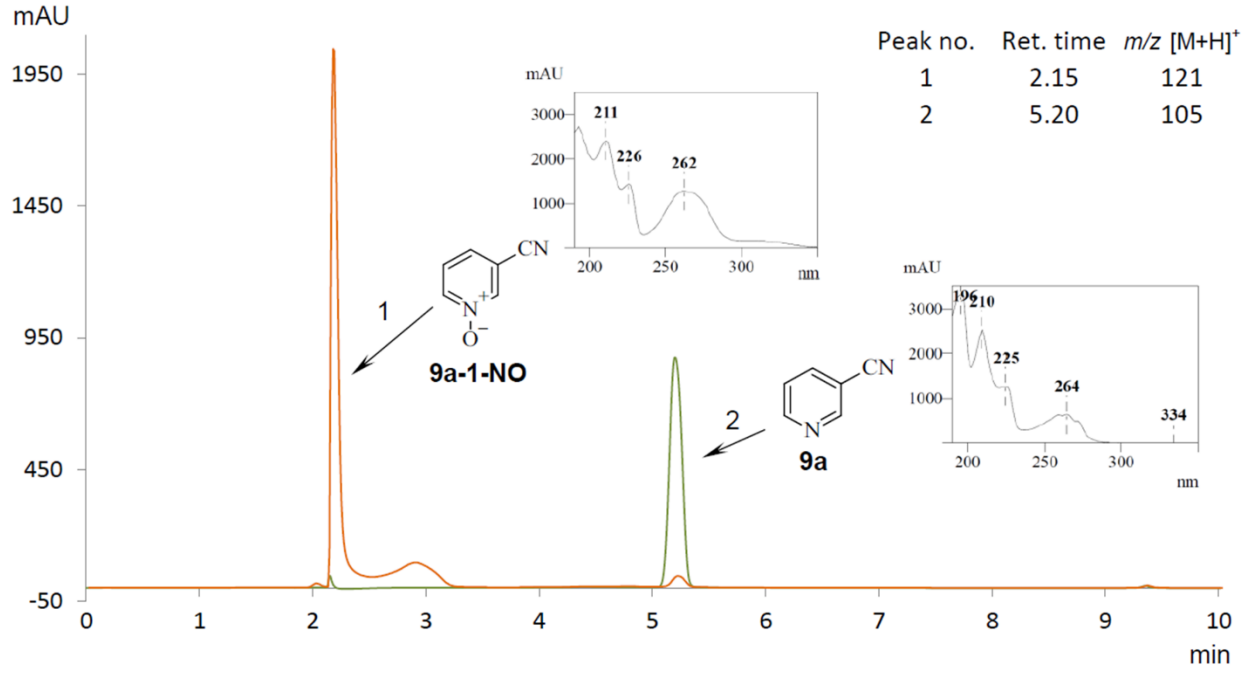
HPLC-MS analysis of 4-methylpyrimidine (**6a**) conversion employing PmlD A113G mutant of PmlABCDEF.



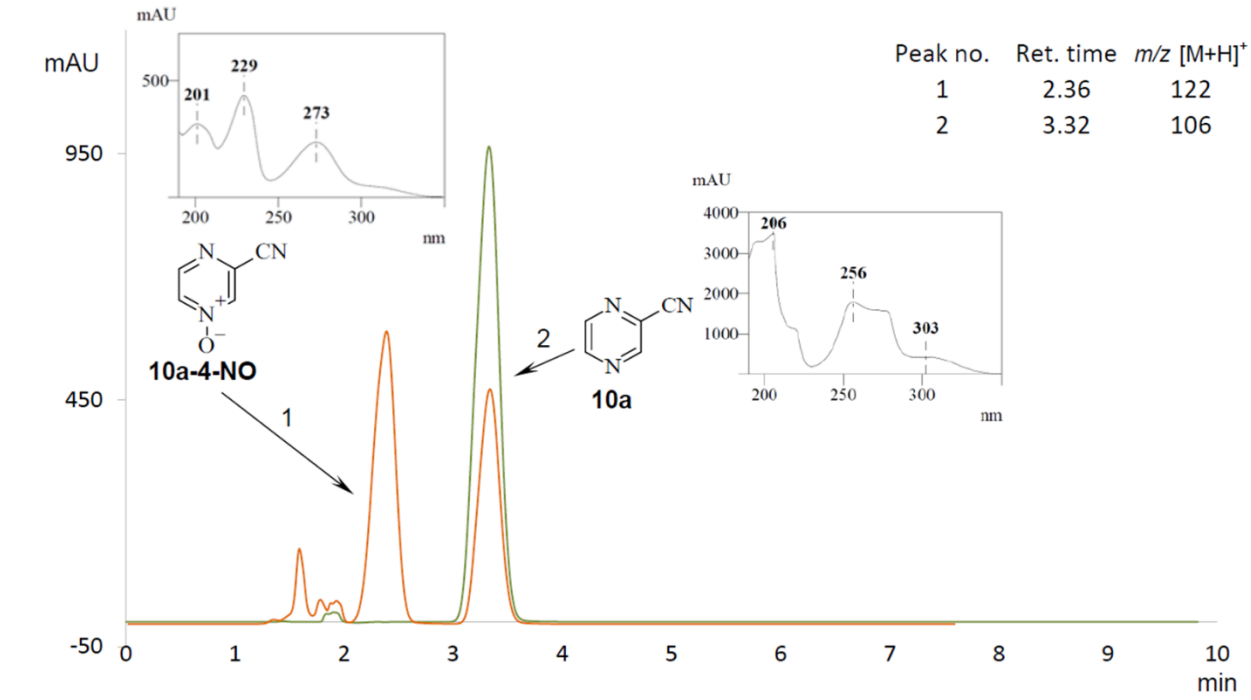
HPLC-MS analysis of 2-chloropyridine (**7a**) conversion employing wild type PmlABCDEF.



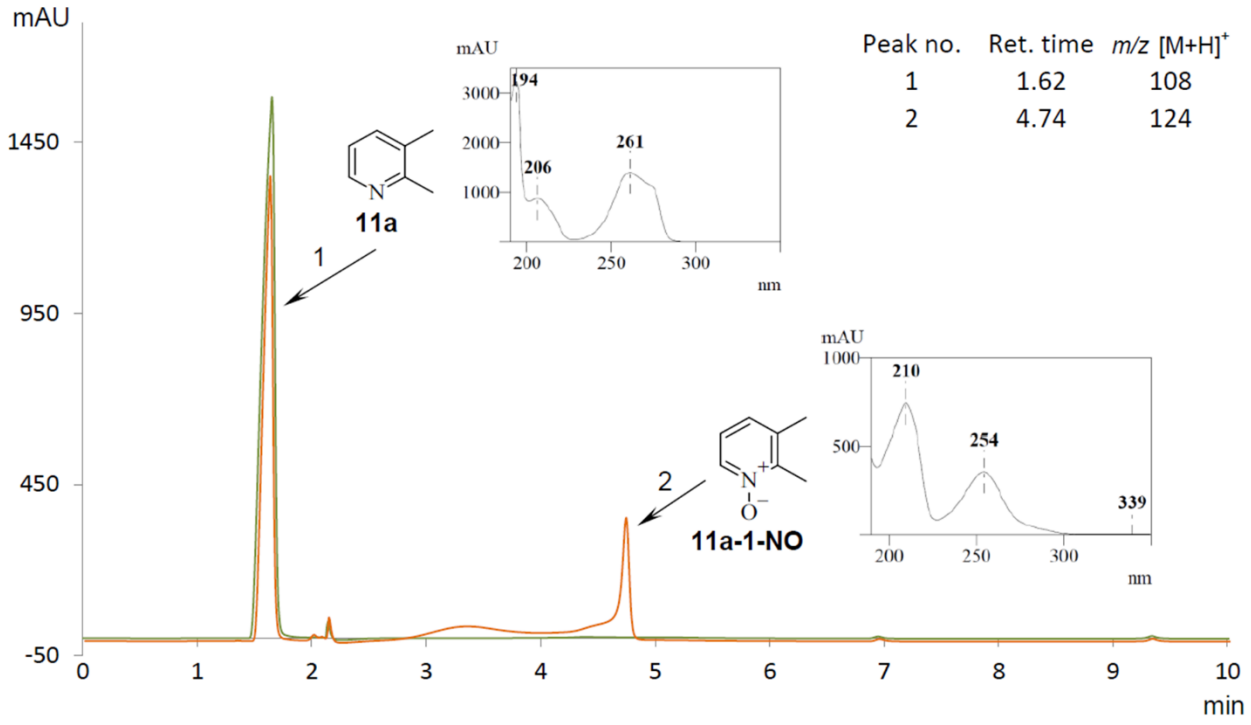
HPLC-MS analysis of 2-chloropyrazine (**8a**) conversion employing wild type PmlABCDEF.



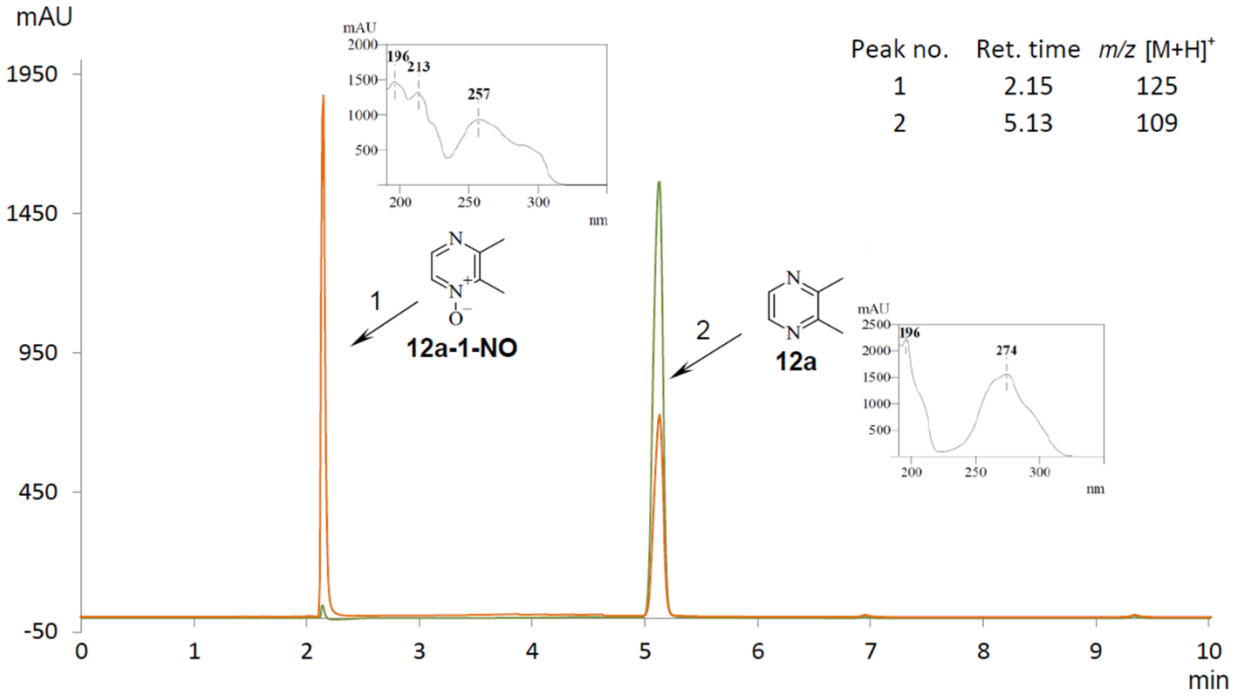
HPLC-MS analysis of 3-cyanopyridine (**9a**) conversion employing wild type PmlABCDEF.



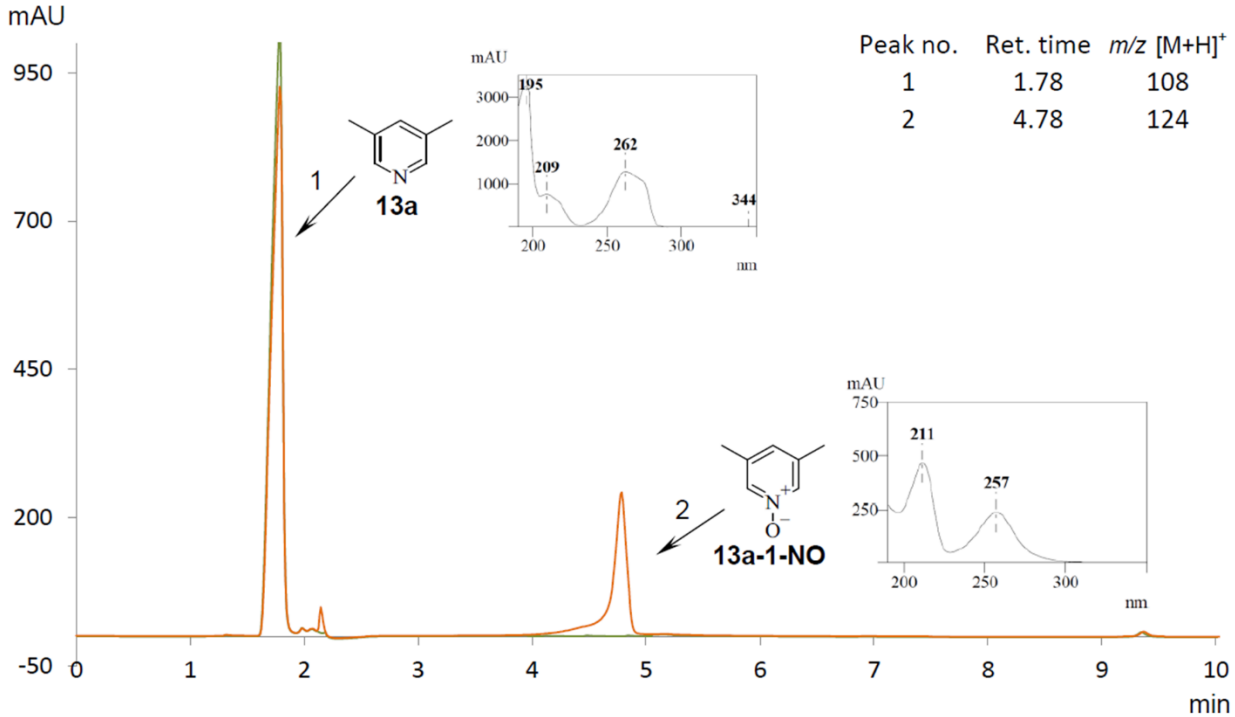
HPLC-MS analysis of 2-cyanopyrazine (**10a**) conversion employing wild type PmlABCDEF.



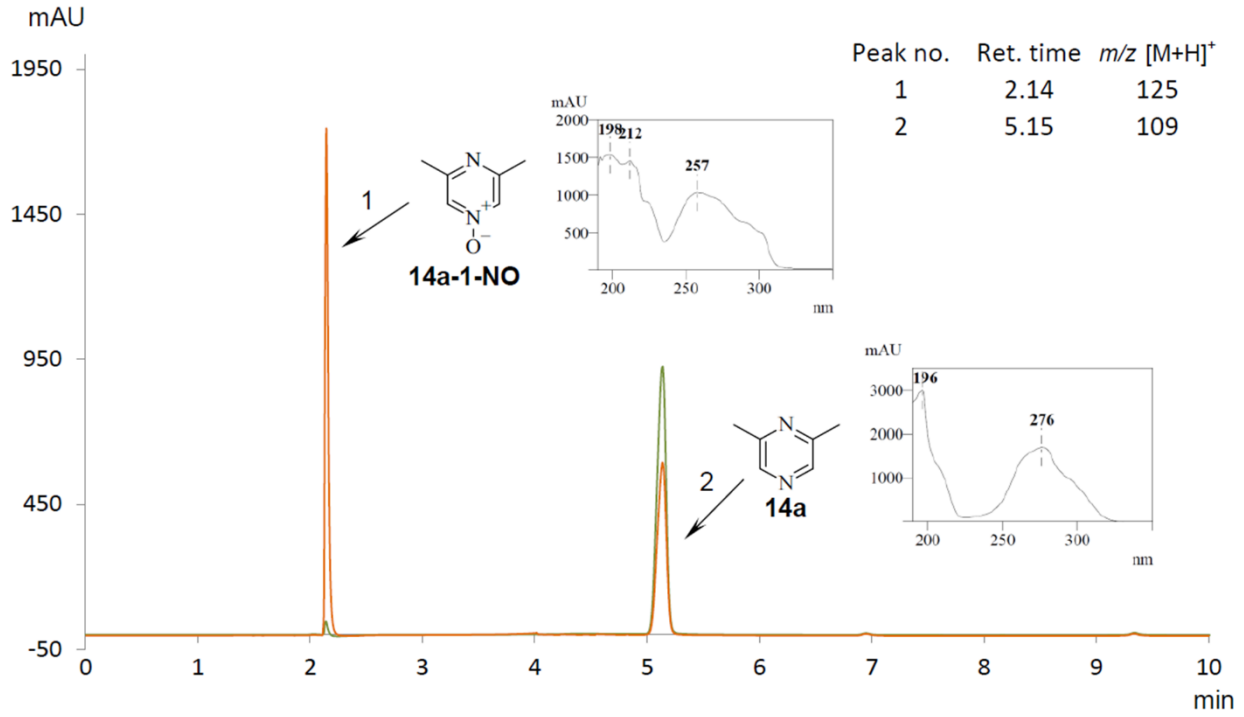
HPLC-MS analysis of 2,3-dimethylpyridine (**11a**) conversion employing wild type PmlABCDEF.



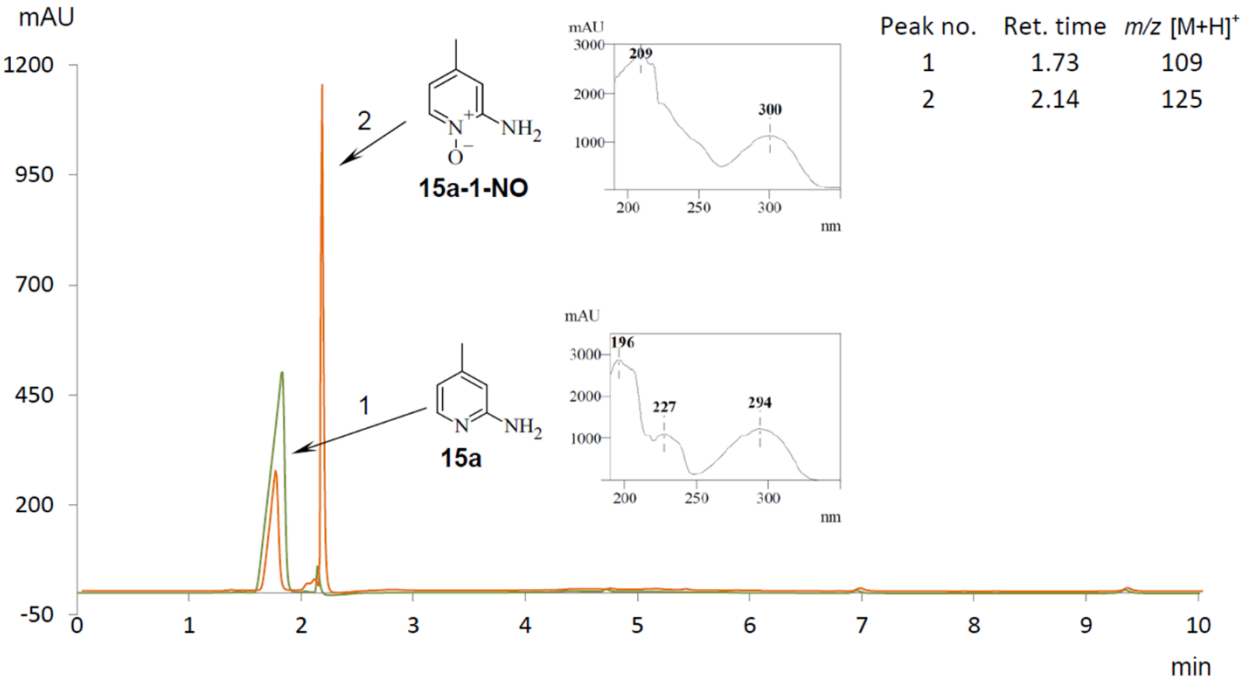
HPLC-MS analysis of 2,3-dimethylpyrazine (**12a**) conversion employing wild type PmlABCDEF.



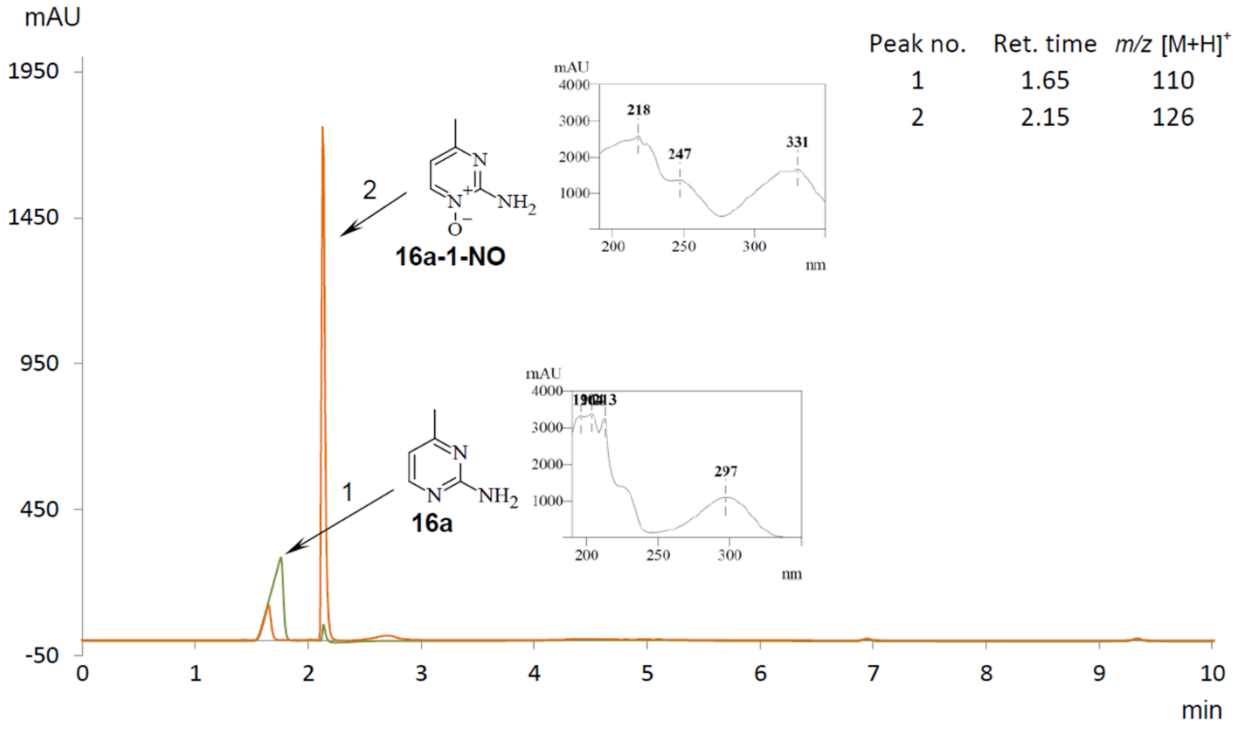
HPLC-MS analysis of 3,5-dimethylpyridine (**13a**) conversion employing wild type PmlABCDEF.



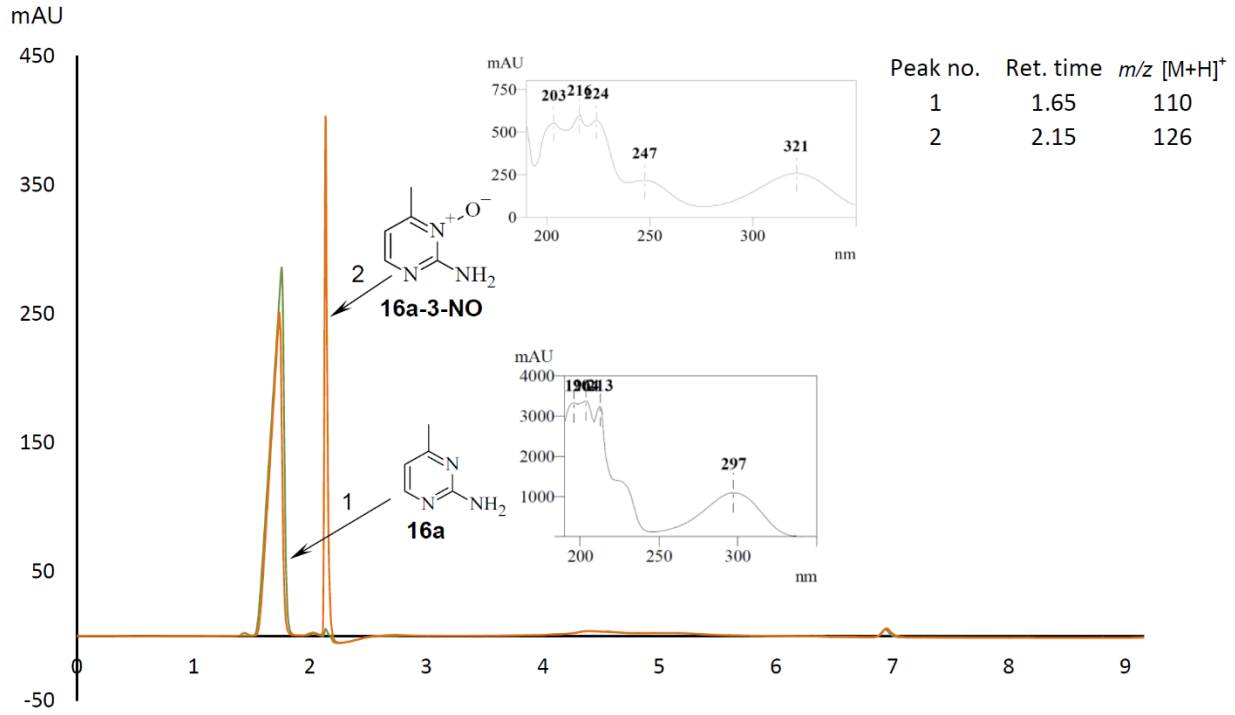
HPLC-MS analysis of 2,6-dimethylpyrazine (**14a**) conversion employing wild type PmlABCDEF.



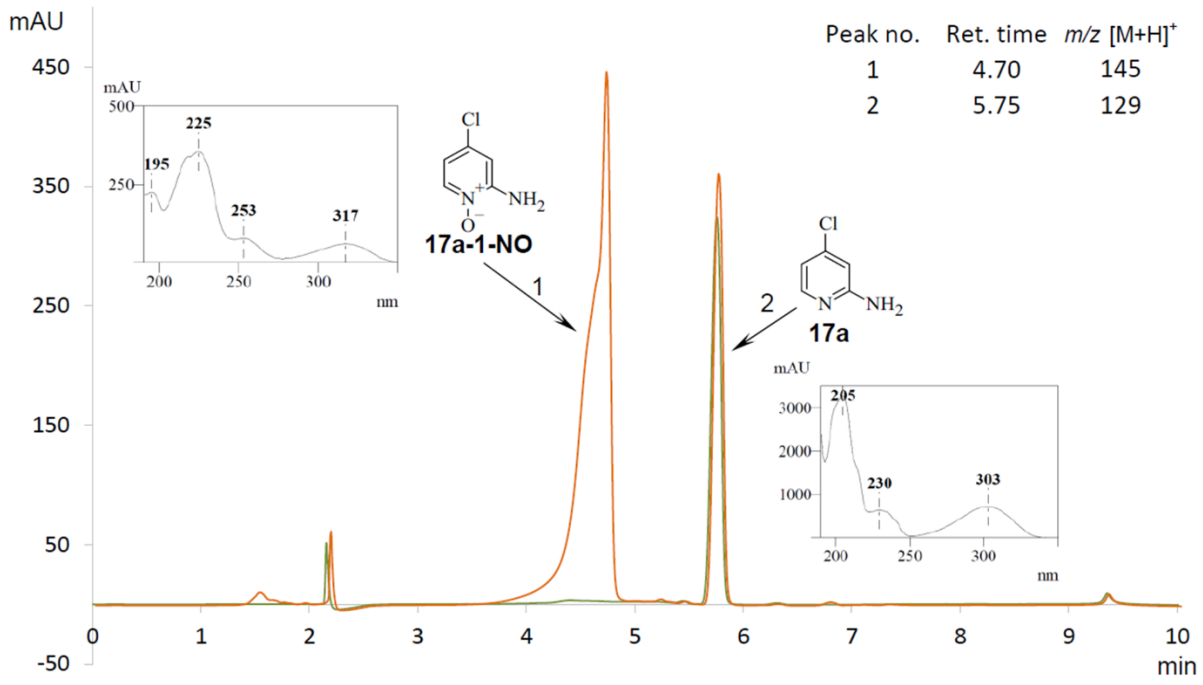
HPLC-MS analysis of 2-amino-4-methylpyridine (**15a**) conversion employing wild type PmlABCDEF.



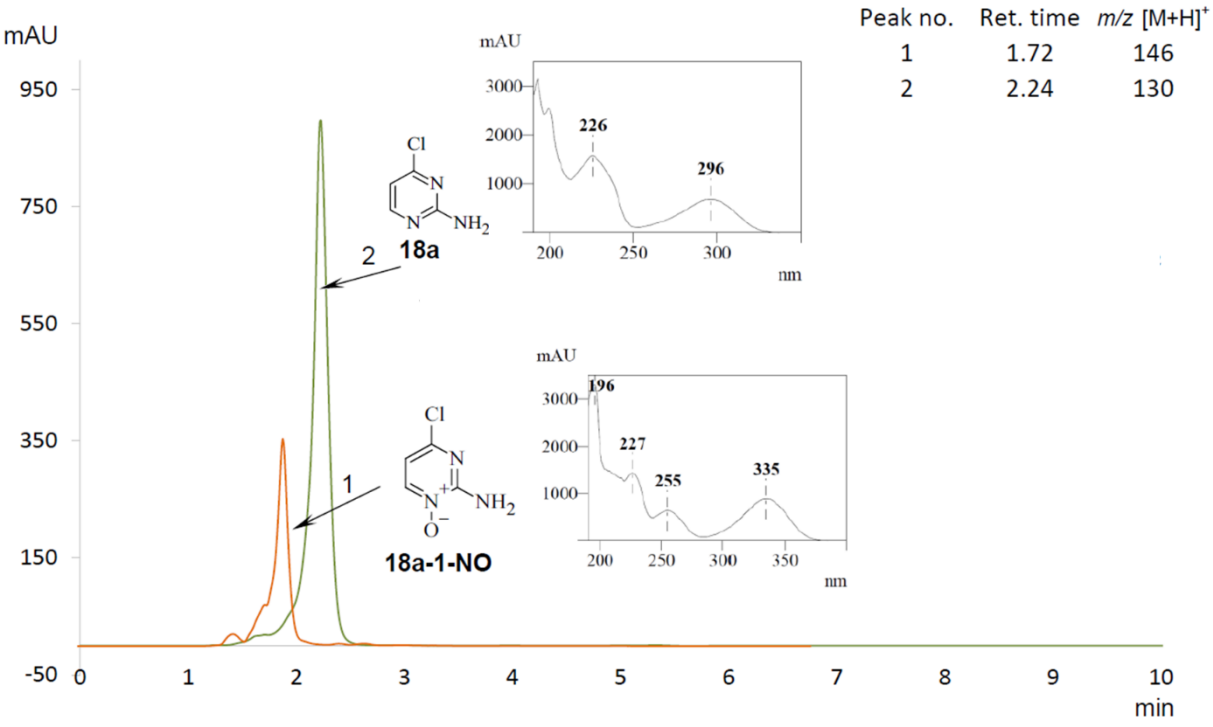
HPLC-MS analysis of 2-amino-4-methylpyrimidine (**16a**) conversion employing wild type PmlABCDEF.



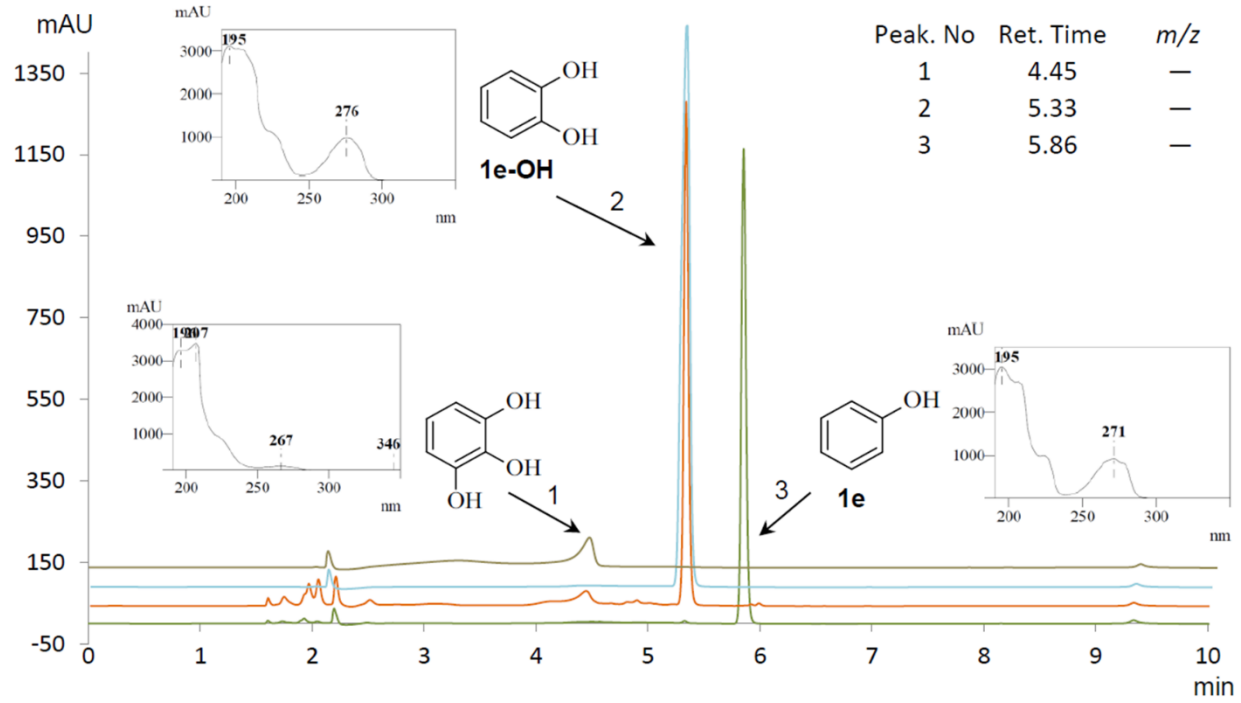
HPLC-MS analysis of 2-amino-4-methylpyrimidine (**16a**) conversion employing PmlD A113G mutant of PmlABCDEF.



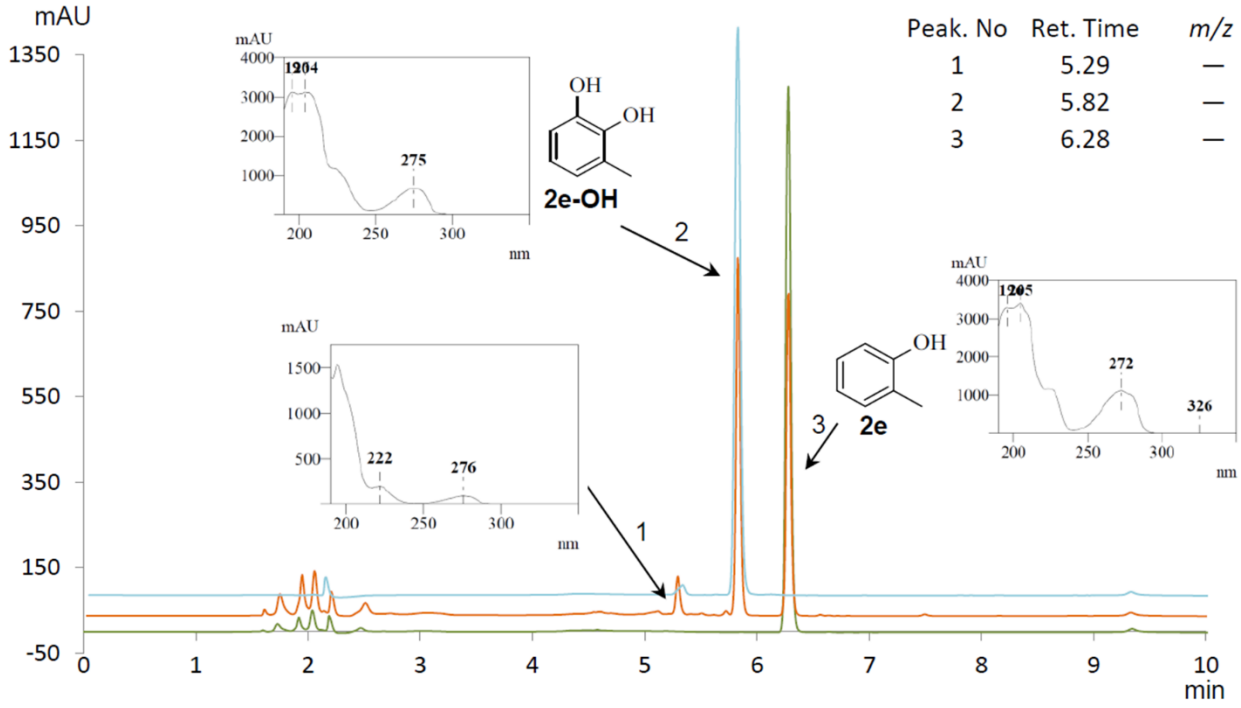
HPLC-MS analysis of 2-amino-4-chloropyridine (**17a**) conversion employing wild type PmlABCDEF.



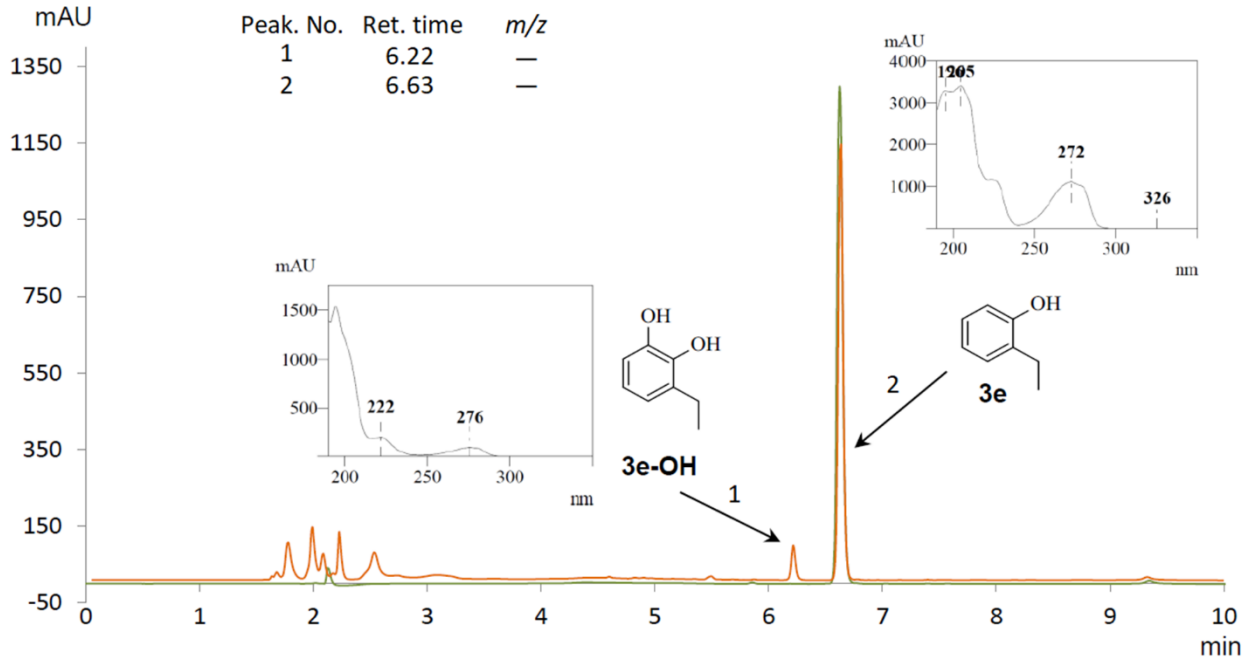
HPLC-MS analysis of 2-amino-4-chloropyrimidine (**18a**) conversion employing wild type PmlABCDEF.



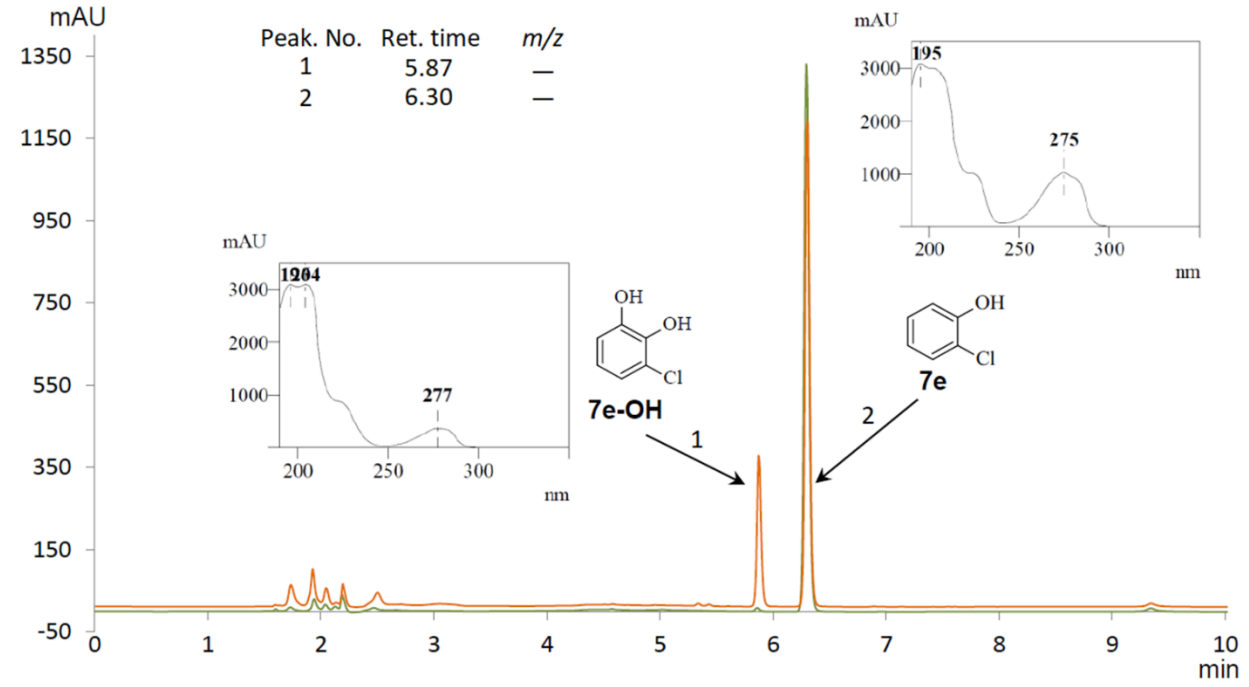
HPLC-MS analysis of phenol (**1e**) conversion employing wild type PmlABCDEF.



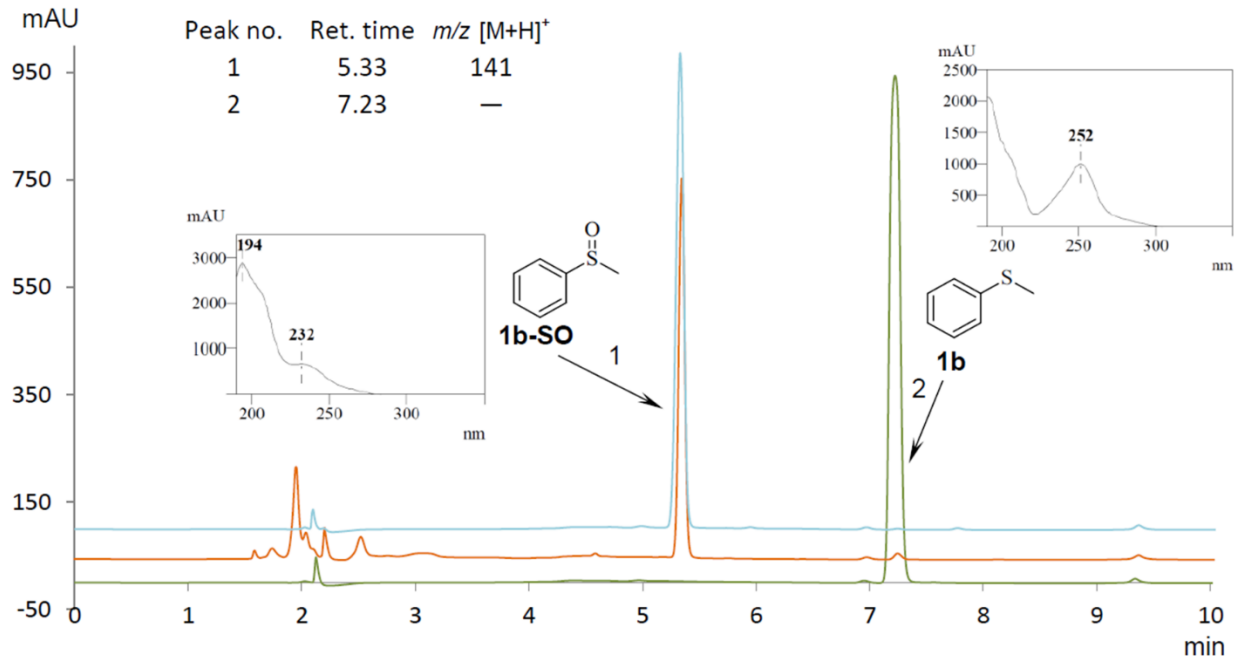
HPLC-MS analysis of 2-methylphenol (**2e**) conversion employing wild type PmlABCDEF.



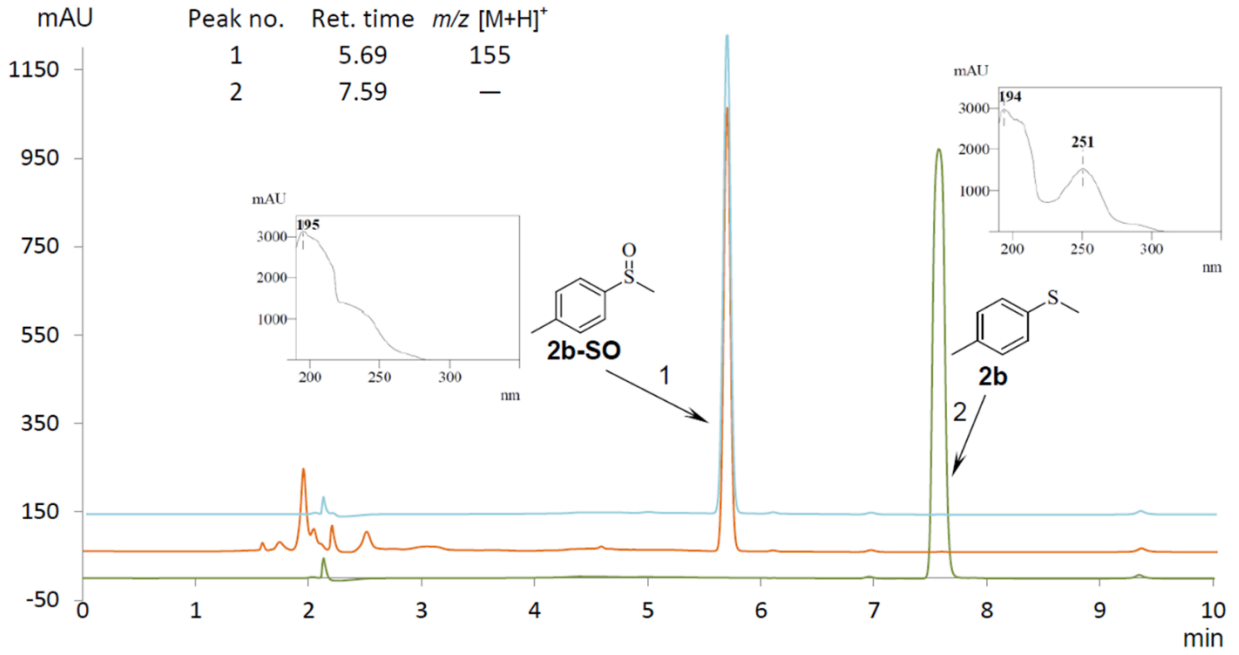
HPLC-MS analysis of 2-ethylphenol (**3e**) conversion employing wild type PmlABCDEF.



HPLC-MS analysis of 2-chlorophenol (**7e**) conversion employing wild type PmlABCDEF.



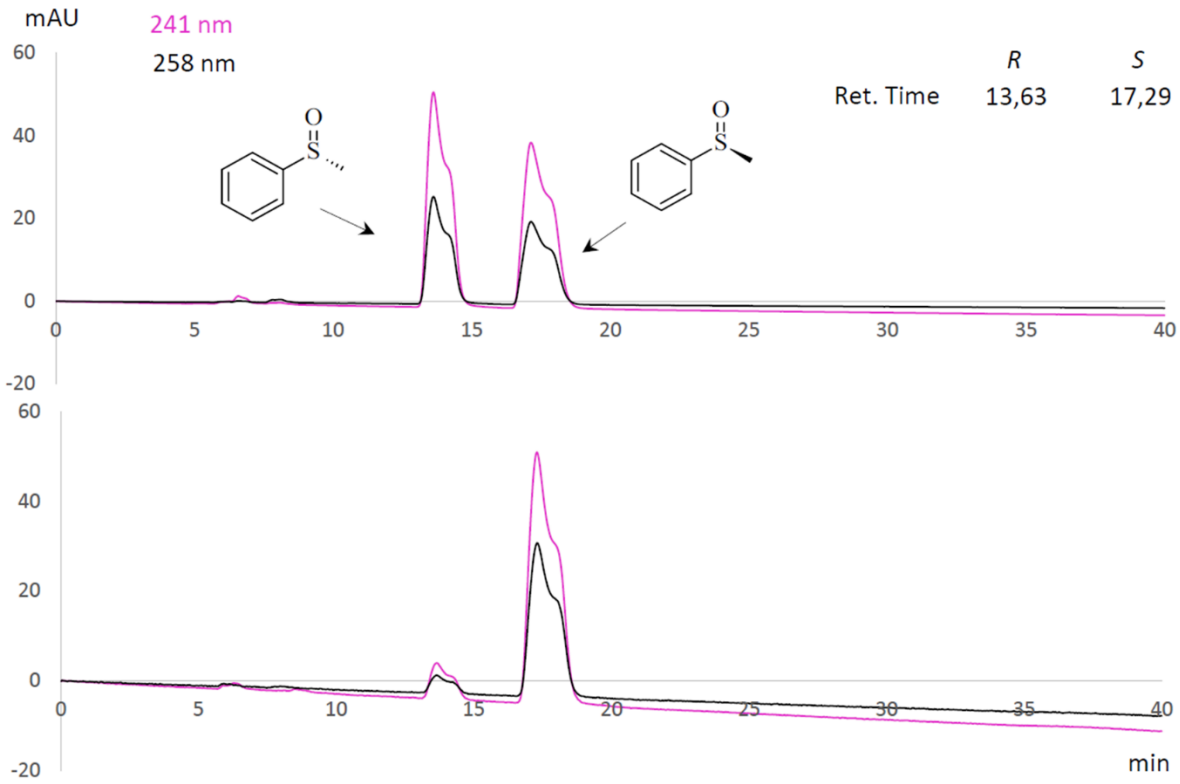
HPLC-MS analysis of methyl phenyl sulfide (**1b**) conversion employing wild type PmlABCDEF.



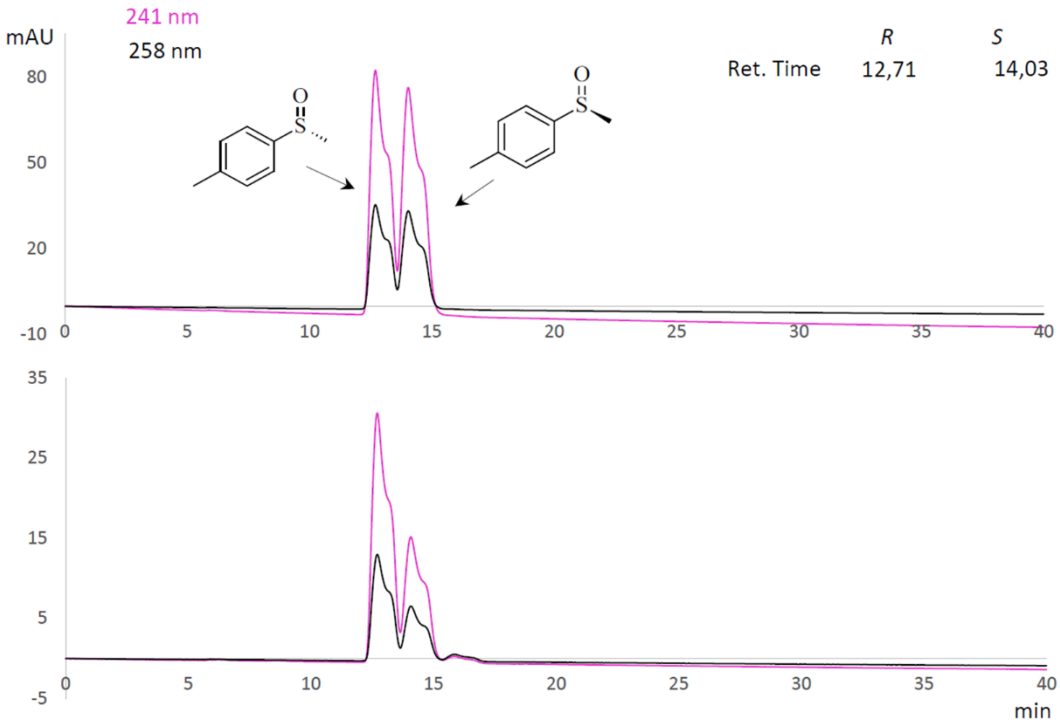
HPLC-MS analysis of methyl *p*-tolyl sulfide (**2b**) conversion employing wild type PmlABCDEF.

Chromatograms of chiral HPLC analysis

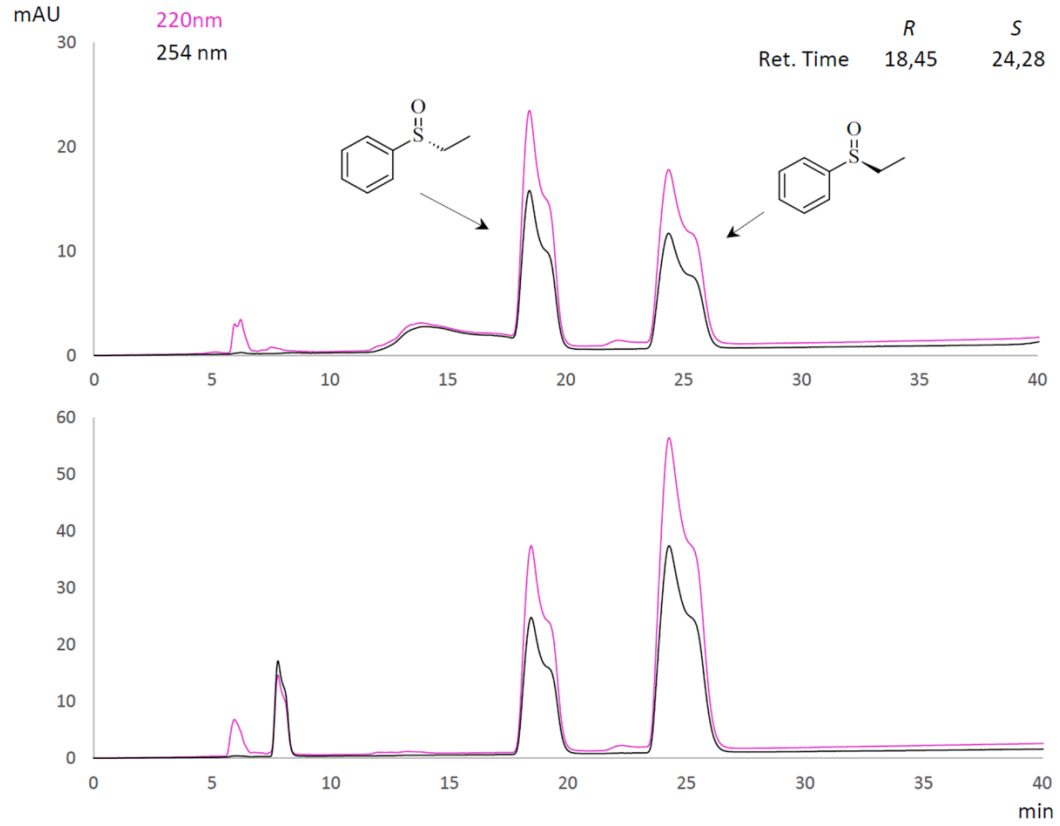
All analyses were done on CHIRALCEL OD-H, 250 x 4.6 mm, 5 µm column (Daicel, Japan). Hexane/ 2-propanol = 90 : 10 system was used, flow rate 0.5 ml min^{-1}. Stacked chromatograms were generated using wavelengths of 241 nm and 220 nm (pink lines); 258 nm and 254 (black lines). The upper panels show chromatograms of appropriate analytical standards, while the lower panels depict end products of Pml-catalysed bioconversions. The absolute configuration was determined by comparison of the optical rotation with the literature value.



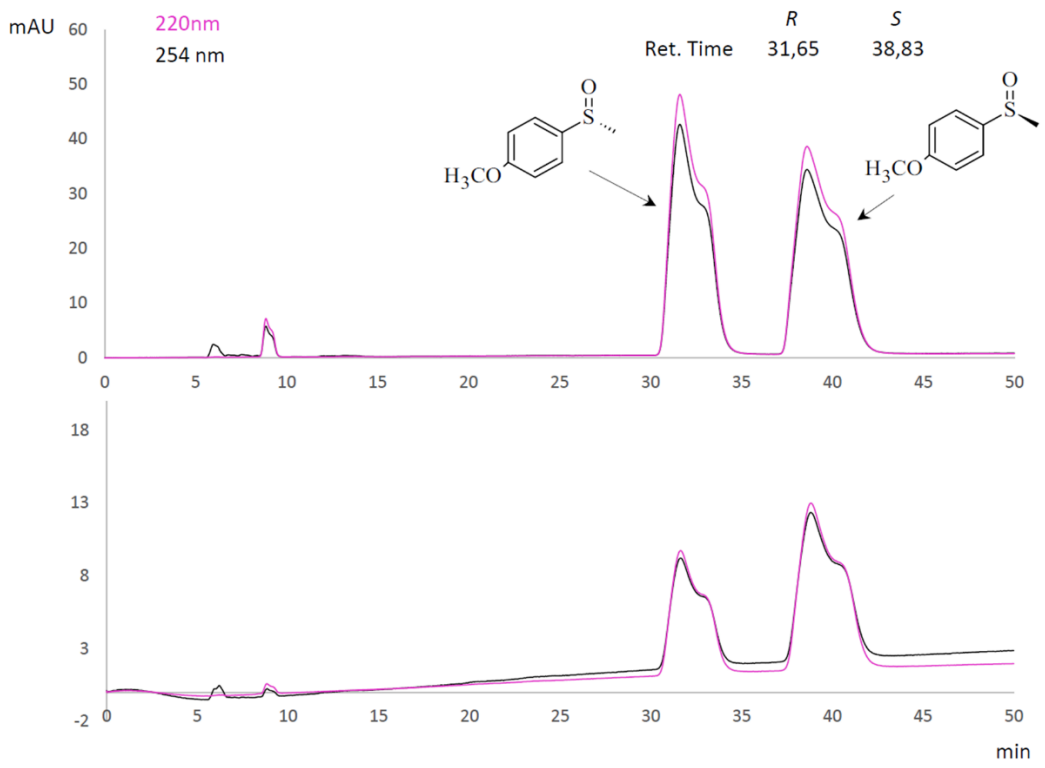
Chiral HPLC analysis of methyl phenyl sulfoxide (**1b-SO**) obtained by wild type PmlABCDEF.



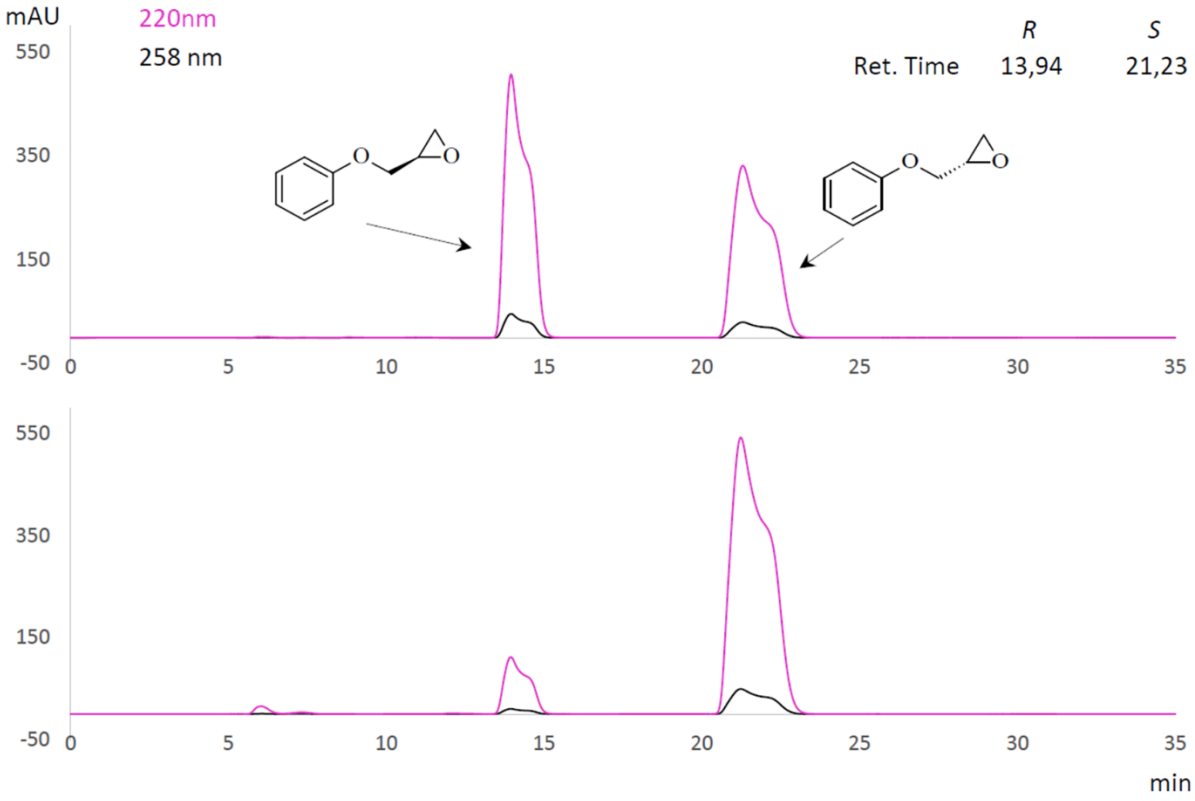
Chiral HPLC analysis of methyl *p*-tolyl sulfoxide (**2b-SO**) obtained by wild type PmlABCDEF.



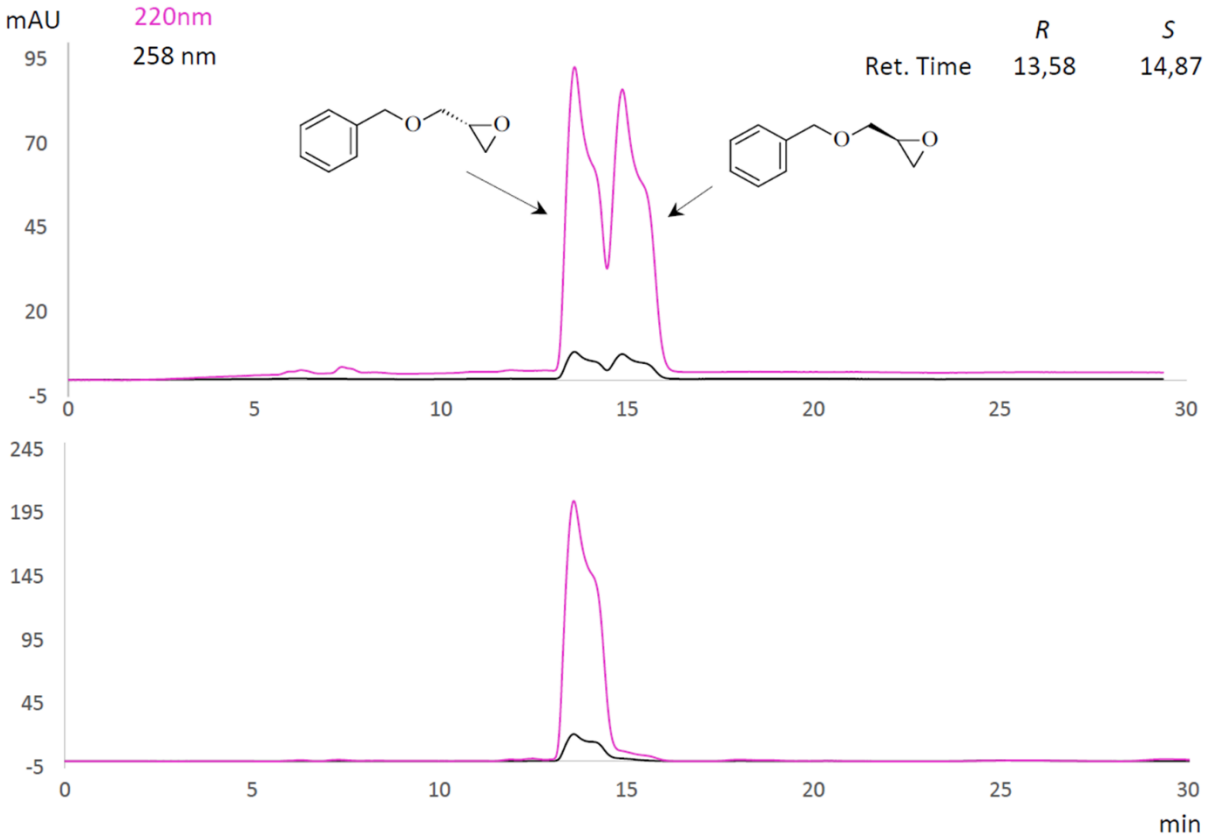
Chiral HPLC analysis of ethyl phenyl sulfoxide (**3b-SO**) obtained by wild type PmlABCDEF.



Chiral HPLC analysis of 4-methoxyphenyl methyl sulfoxide (**5b-SO**) obtained by wild type PmlABCDEF.



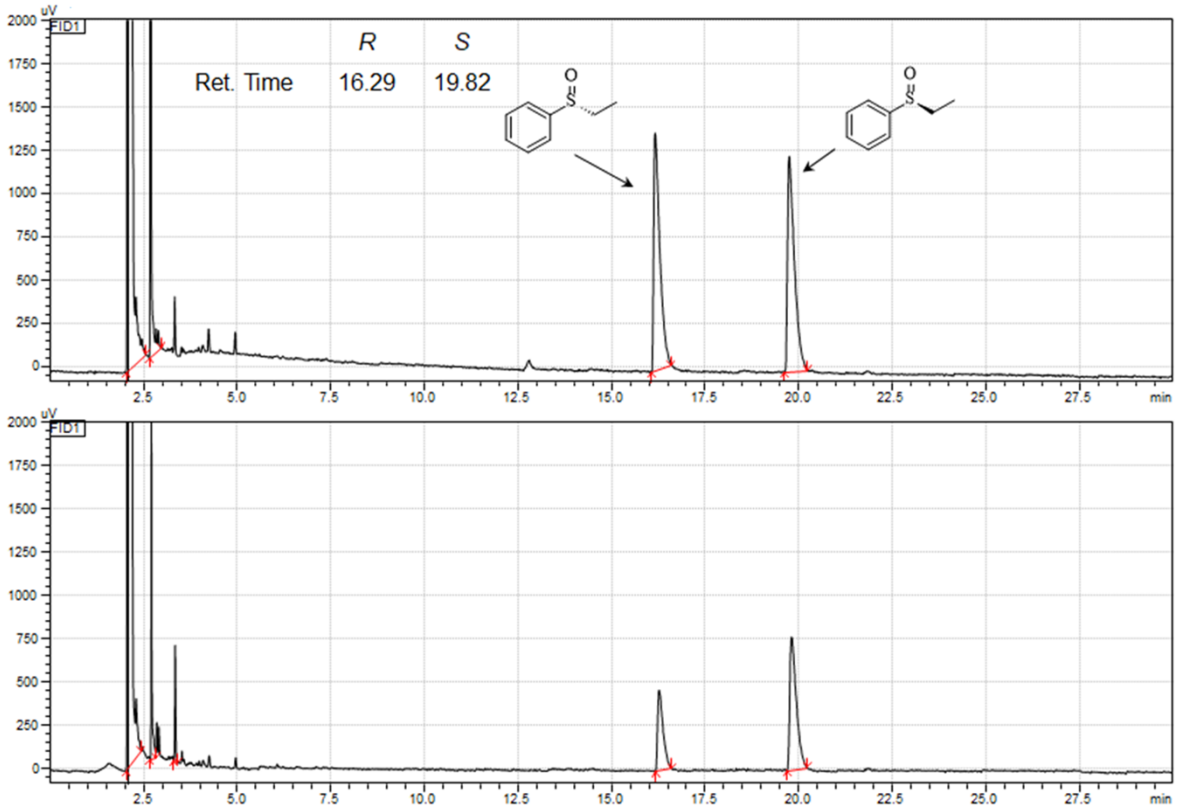
Chiral HPLC analysis of glycidyl phenyl ether (**8c-OX**) obtained by wild type PmlABCDEF.



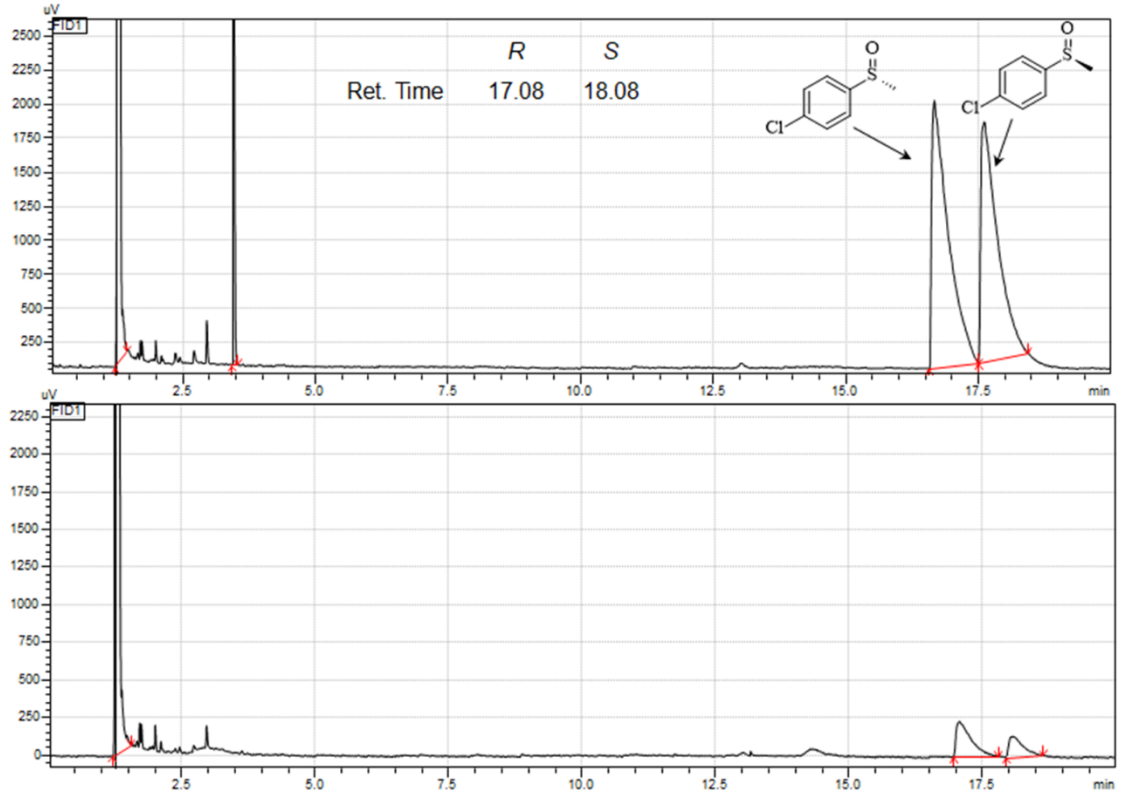
Chiral HPLC analysis of glycidyl benzyl ether (**9c-OX**) obtained by wild type PmlABCDEF.

Chromatograms of chiral GC analysis

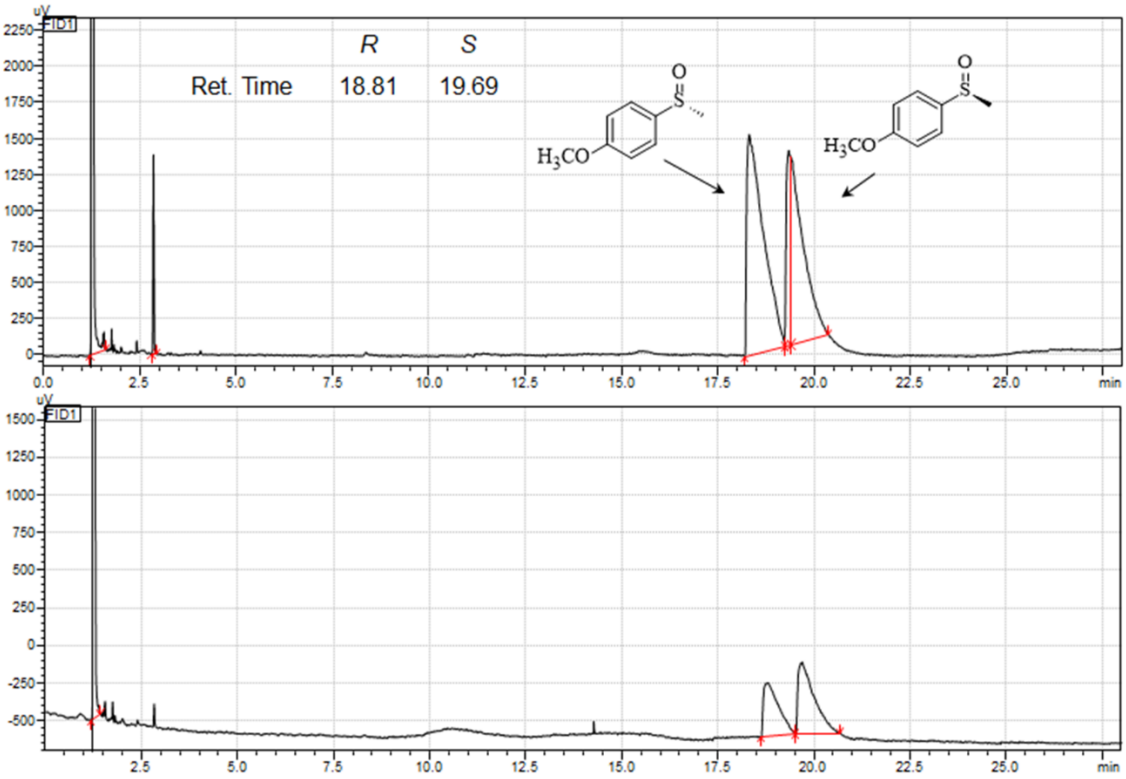
The measurements were done with an Astec Chiraldex G-PN Column (length 30 mm, inner diameter 0.25 mm, film thickness 0.12 µm) from Sigma-Aldrich, Germany. Helium was used as a carrier gas with a linear velocity of 50 cm sec^{-1}. The injection volume was 1 µL with an injection port temperature of 350 °C and a split ratio of 1:200. The upper panels show chromatograms of appropriate analytical standards, while the lower panels depict end products of Pml-catalysed bioconversions. The absolute configuration was determined by comparison of the optical rotation with the literature value.



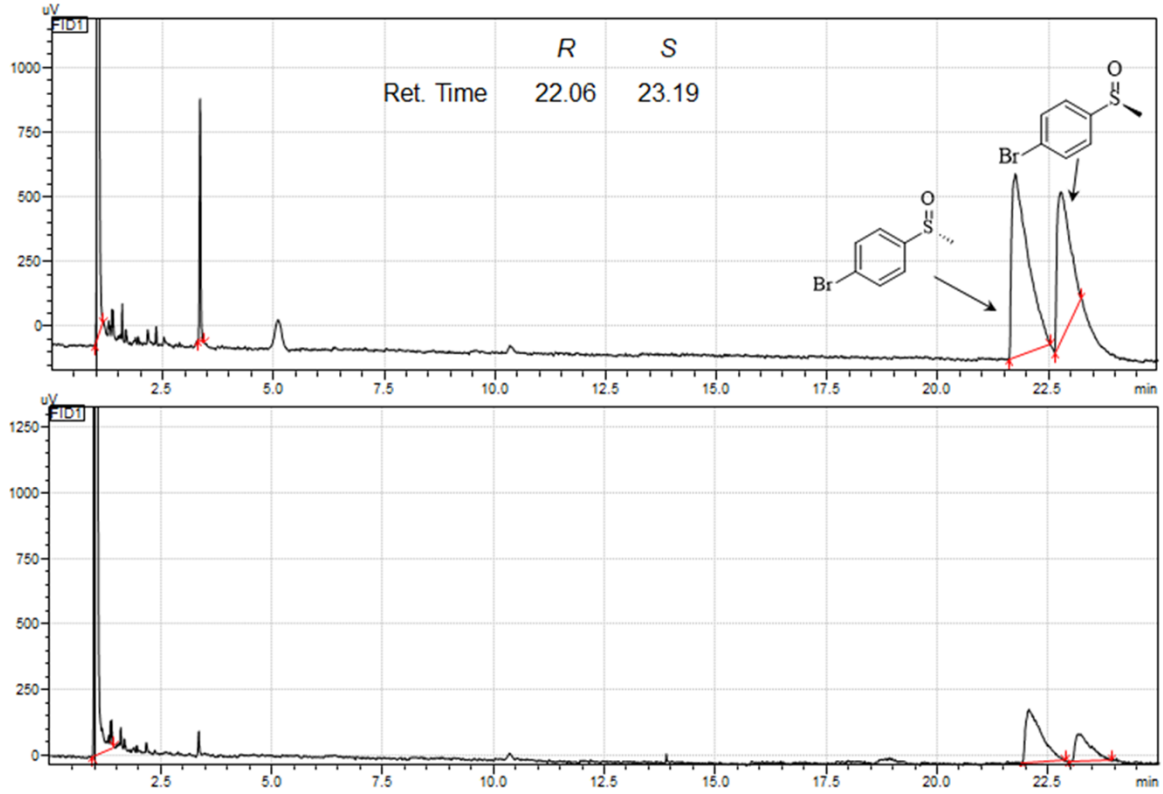
Chiral GC analysis of ethyl phenyl sulfoxide (**3b-SO**) obtained by wild type PmlABCDEF.



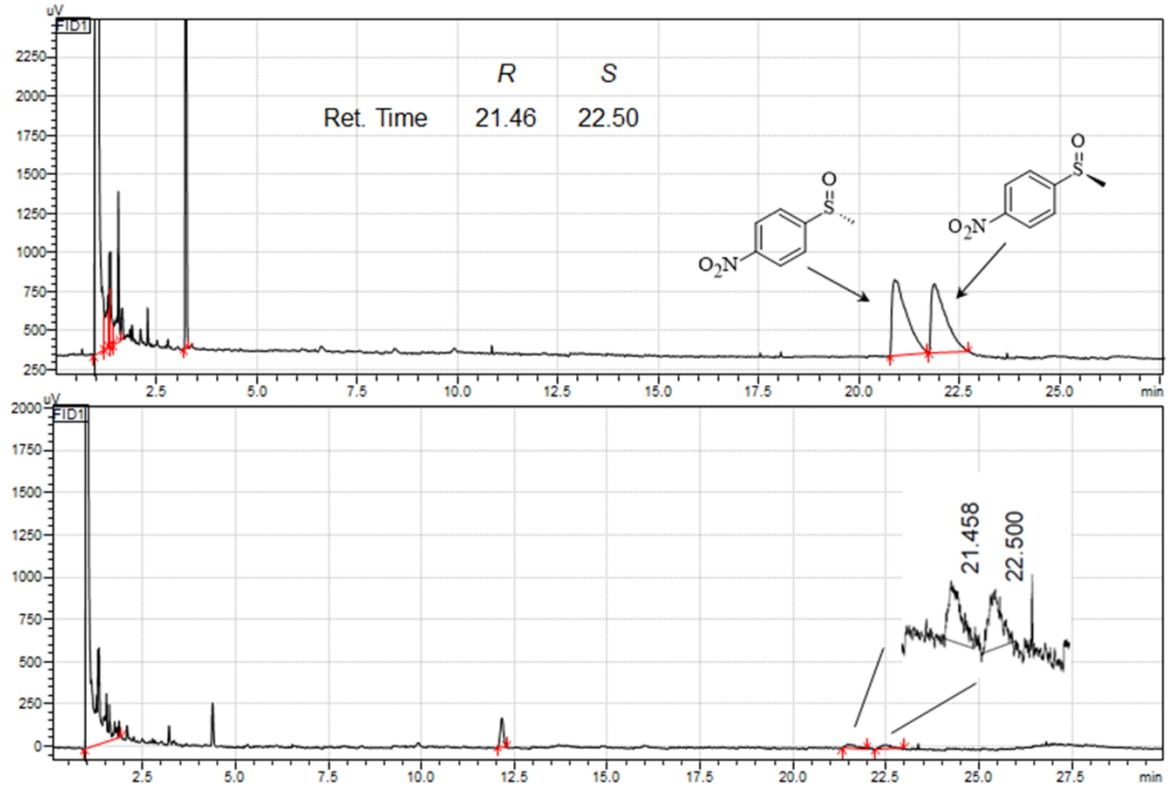
Chiral GC analysis of methyl 4-chlorophenyl sulfoxide (**4b-SO**) obtained by wild type PmlABCDEF.



Chiral GC analysis of 4-methoxyphenyl methyl sulfoxide (**5b-SO**) obtained by wild type PmlABCDEF.



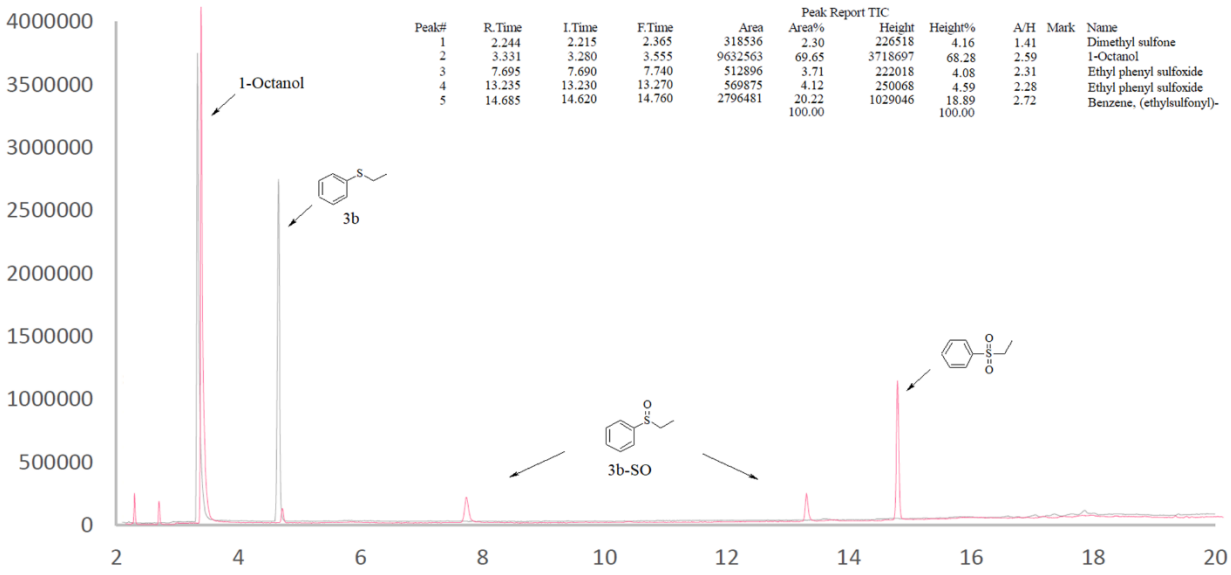
Chiral GC analysis of methyl 4-bromophenyl sulfoxide (**6b-SO**) obtained by wild type PmlABCDEF.



Chiral GC analysis of methyl 4-nitrophenyl sulfoxide (**7b-SO**) obtained by wild type PmlABCDEF

Chromatograms of GC-MS analysis

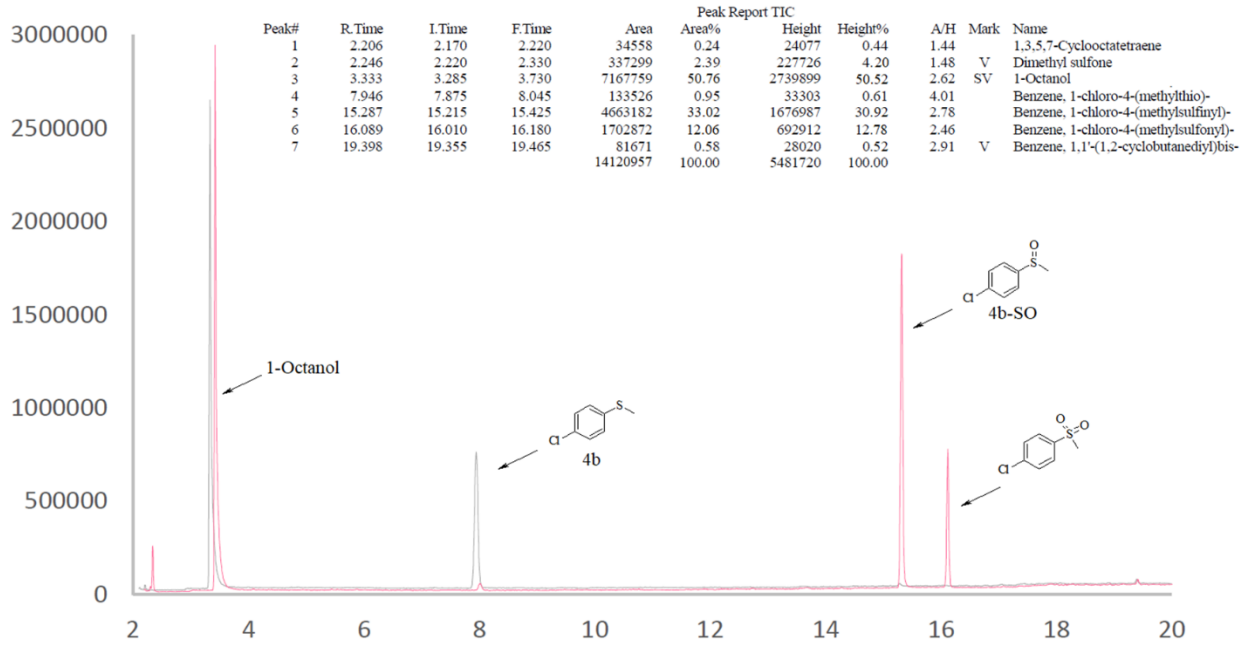
The sample (0.5 mL) of the whole-cell biocatalysis reaction was transferred to a 1.5 mL tube and mixed with an equal part of ethyl acetate (dichloromethane for styrene). The mixture was intensively shaken followed by centrifugation (12000 *g* for 5 min), 0.5 mL of organic phase was dried over $MgSO_4$ or Na_2SO_4 and analysed with GC-MS. Stacked GC chromatograms were designated as follows: i) substrate incubated with *P. putida* KT2440 cells (control, grey line) ii) substrate incubated with *P. putida* KT2440 cells producing Pml monooxygenase (reaction, pink line). A peak report table of the reaction sample is displayed. 1-Octanol was used as an internal standard in all samples except styrene.



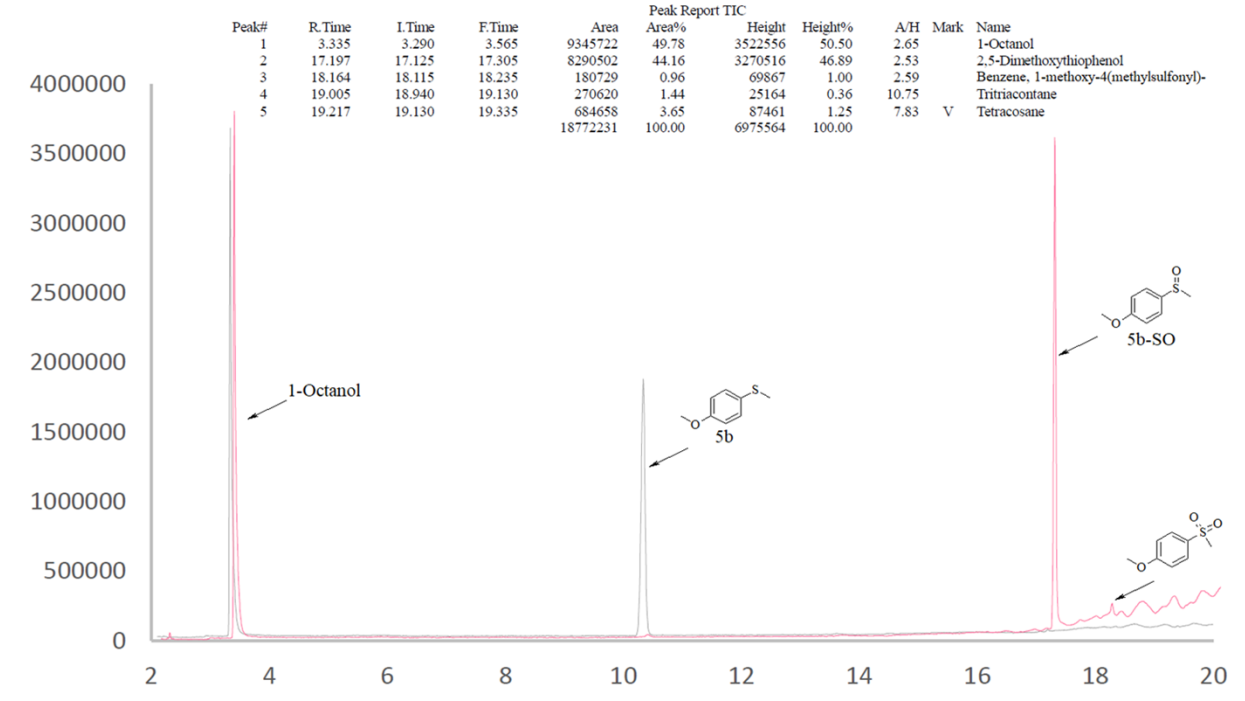
Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	2.244	2.215	2.365	318536	2.30	226518	4.16	1.41		Dimethyl sulfone
2	3.331	3.280	3.555	9632563	69.65	3718697	68.28	2.59		1-Octanol
3	7.695	7.690	7.740	512896	3.71	222018	4.08	2.31		Ethyl phenyl sulfoxide
4	13.235	13.230	13.270	569875	4.12	250068	4.59	2.28		Ethyl phenyl sulfoxide
5	14.685	14.620	14.760	2796481	20.22	1029046	18.89	2.72		Benzene, (ethylsulfonyl)-
					100.00		100.00			

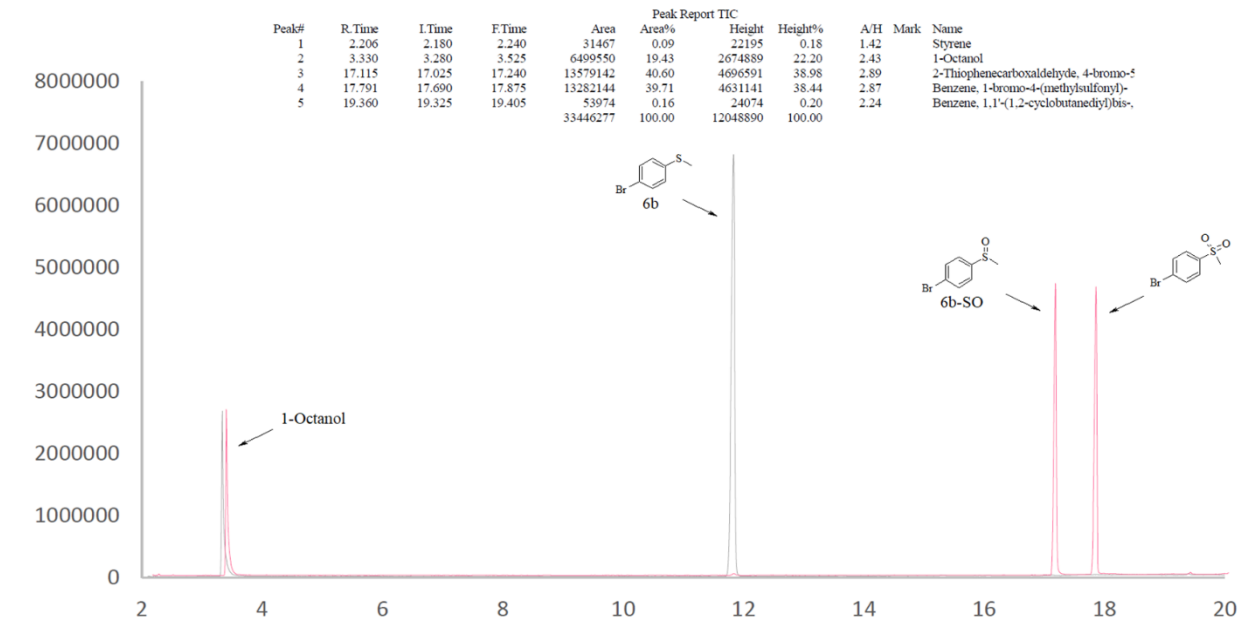
GC-MS analysis of ethyl phenyl sulfide (**3b**) conversion employing wild type PmlABCDEF.



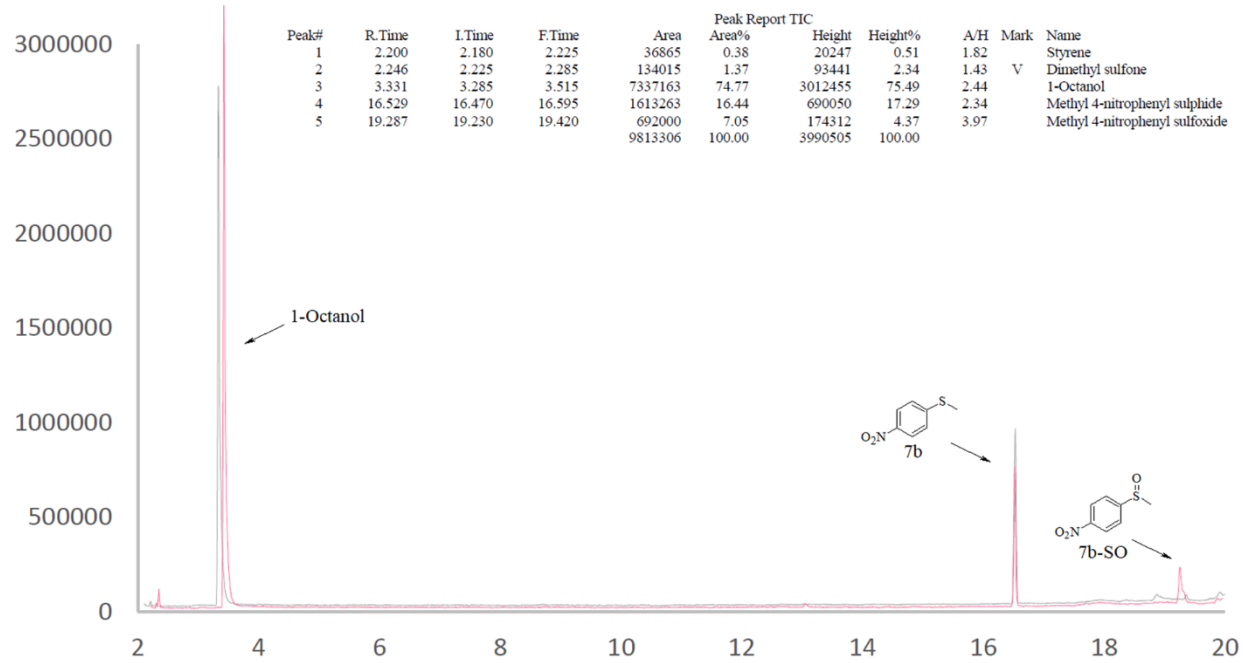
GC-MS analysis of 4-chlorophenyl methyl sulfide (**4b**) conversion employing wild type PmlABCDEF.



GC-MS analysis of 4-methoxyphenylmethyl sulfide (**5b**) conversion employing wild type PmlABCDEF.



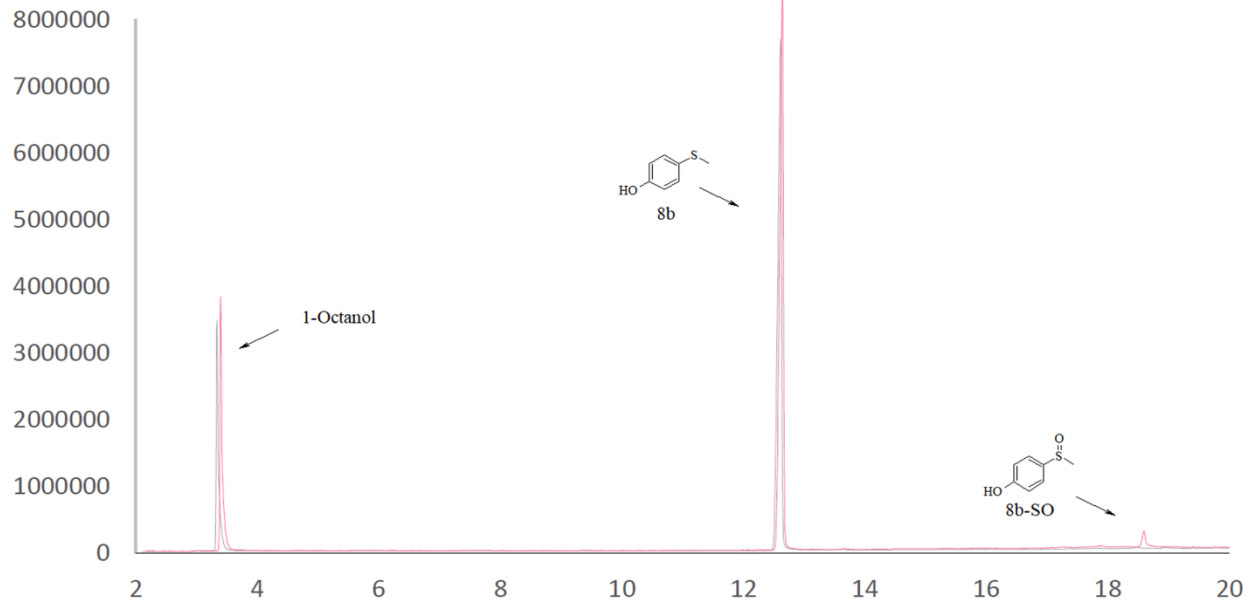
GC-MS analysis of 4-bromophenyl methyl sulfide (**6b**) conversion employing wild type PmlABCDEF.



GC-MS analysis of methyl-4-nitrophenyl sulfide (**7b**) conversion employing wild type PmlABCDEF.

Peak Report TIC

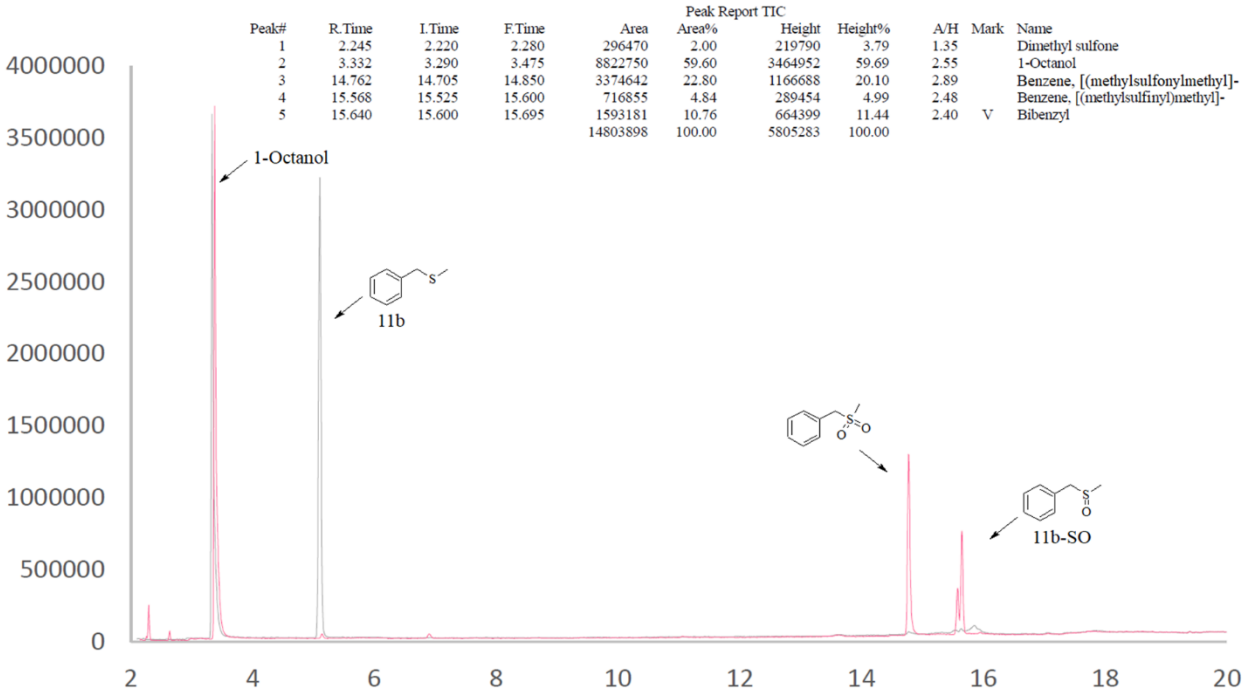
Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	2.206	2.180	2.230	10402	0.03	7996	0.08	1.30		Styrene
2	3.334	3.280	3.555	8443468	23.31	3231575	30.70	2.61		1-Octanol
3	12.614	12.455	12.790	30362388	76.64	7091530	67.37	4.28		Phenol, 4-(methylthio)-
4	18.580	18.490	18.745	800478	2.02	195784	1.86	4.09		Phenol, 2-(methylsulfinyl)-
				39617006	100.00		100.00			



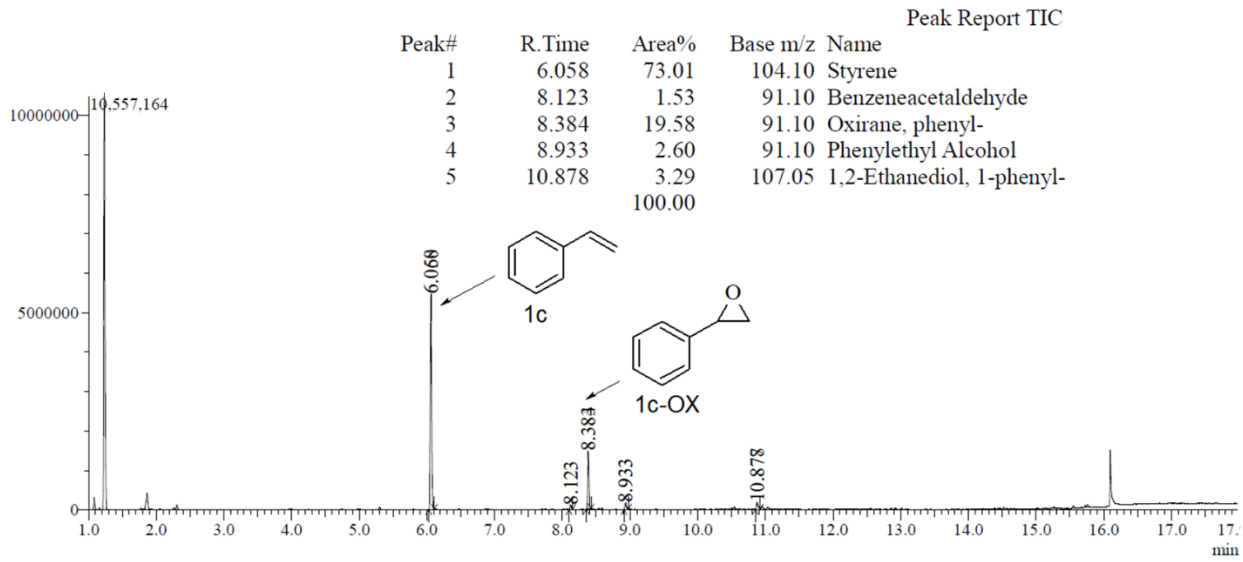
GC-MS analysis of 4-(methylthio)phenol (**8b**) conversion employing wild type PmlABCDEF.

Peak Report TIC

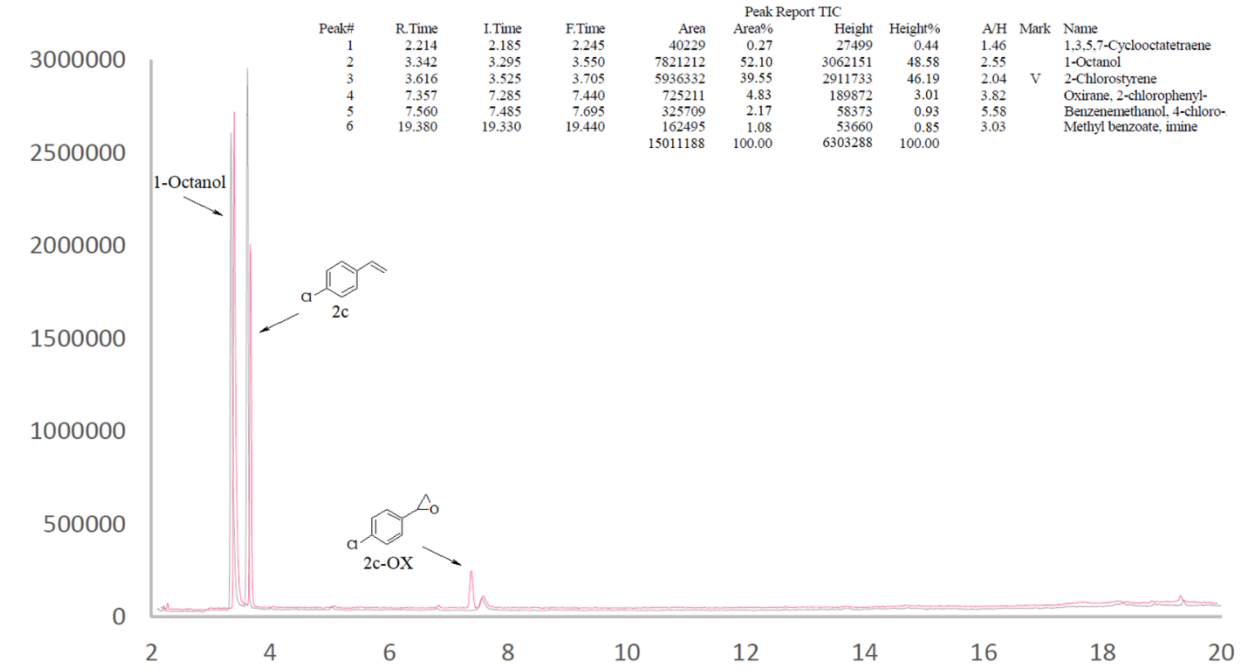
Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	2.245	2.220	2.280	296470	2.00	219790	3.79	1.35		Dimethyl sulfone
2	3.332	3.290	3.475	8822750	59.60	3464952	59.69	2.55		1-Octanol
3	14.762	14.705	14.850	3374642	22.80	1166688	20.10	2.89		Benzene, [(methylsulfonylmethyl]-
4	15.568	15.525	15.600	716855	4.84	289454	4.99	2.48		Benzene, [(methylsulfinyl)methyl]-
5	15.640	15.600	15.695	1593181	10.76	664399	11.44	2.40	V	Bibenzyl
				14803898	100.00	5805283	100.00			



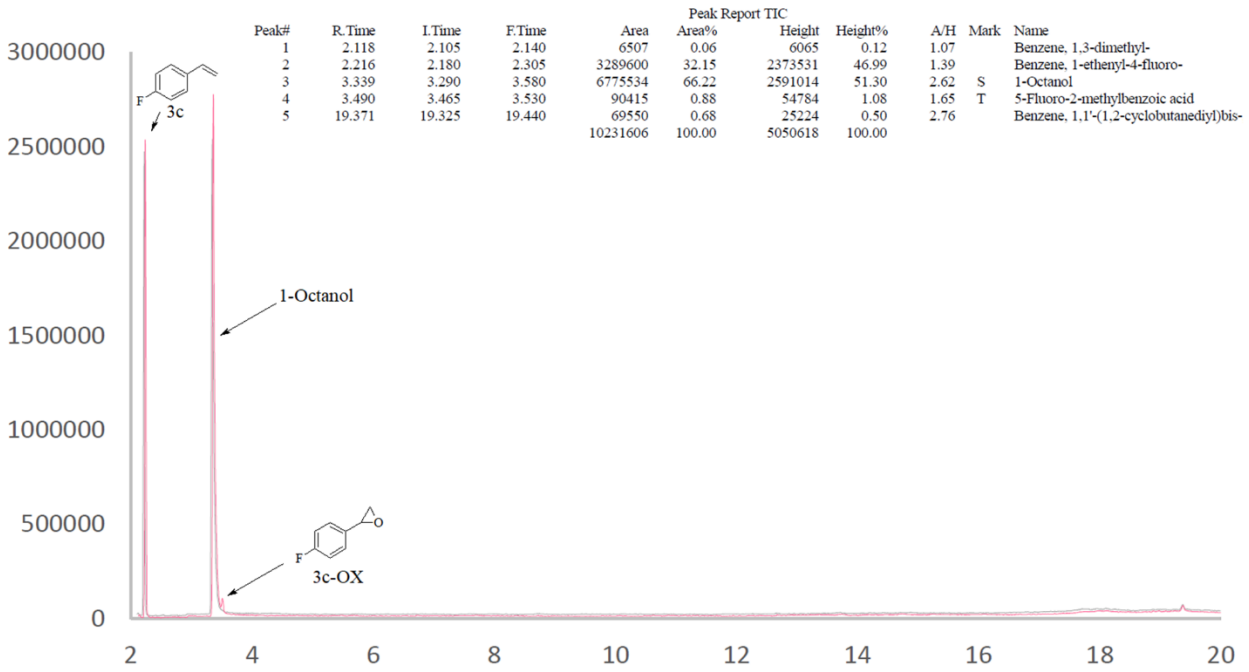
GC-MS analysis of benzyl methyl sulfide (**11b**) conversion employing wild type PmlABCDEF.



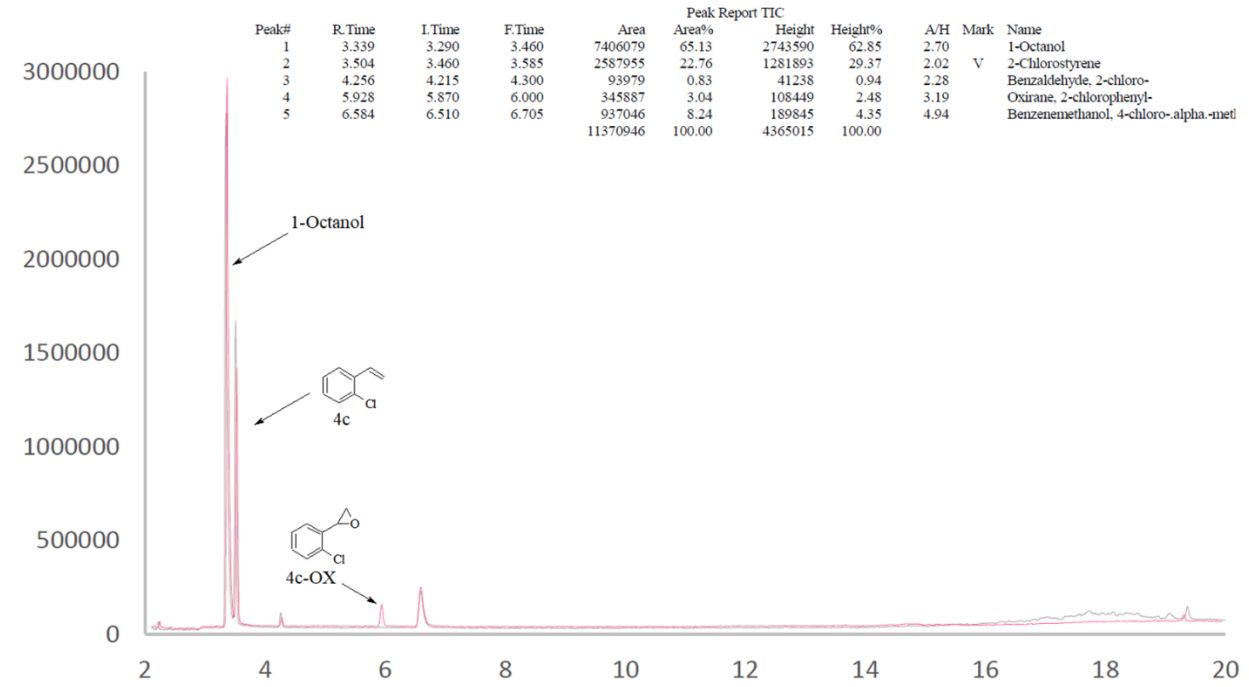
GC-MS analysis of styrene (**1c**) conversion employing wild type PmlABCDEF.



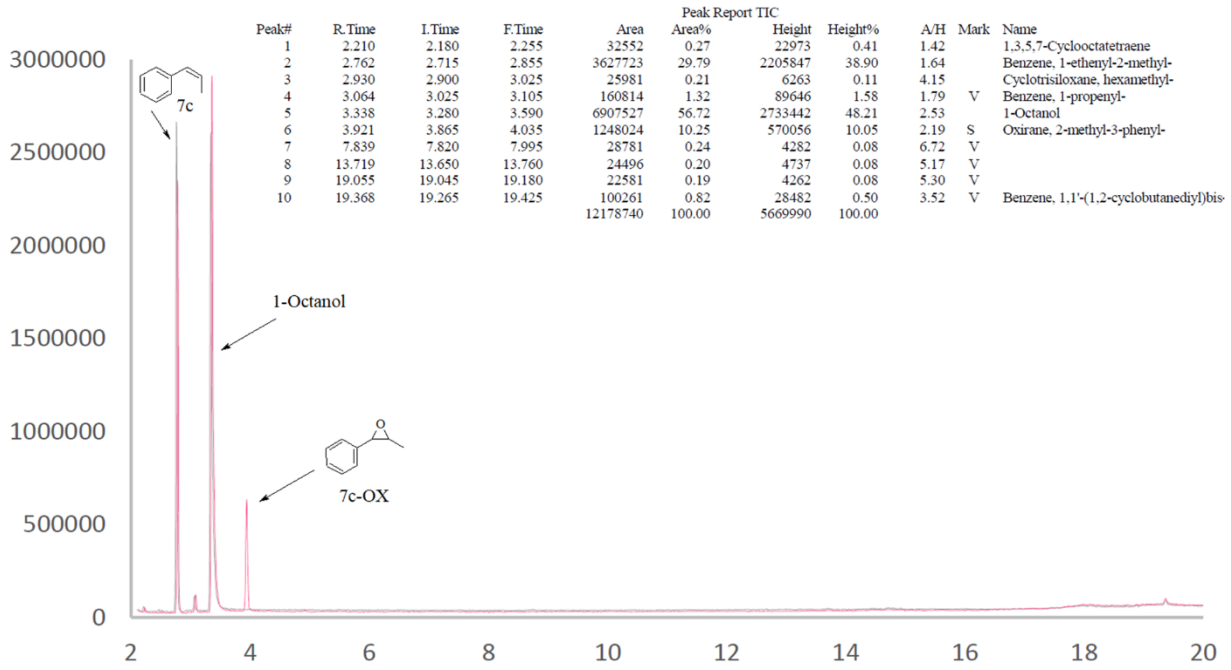
GC-MS analysis of 4-chlorostyrene (**2c**) conversion employing wild type PmlABCDEF.



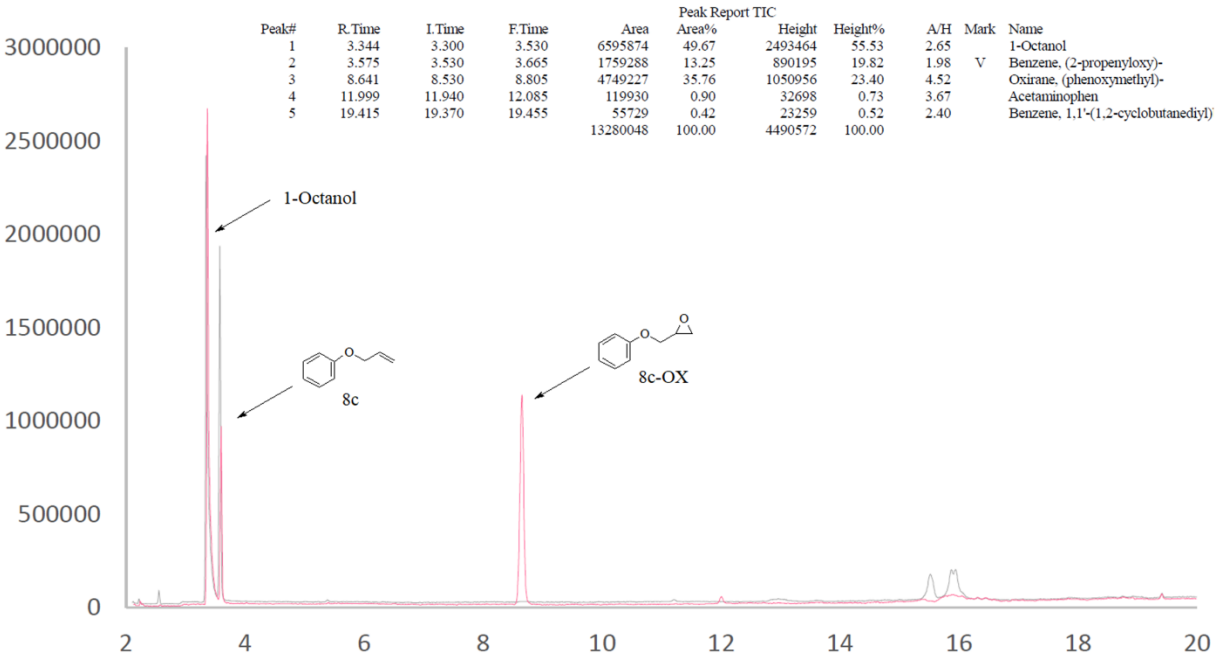
GC-MS analysis of 4-fluorostyrene (**3c**) conversion employing wild type PmlABCDEF.



GC-MS analysis of 2-chlorostyrene (**4c**) conversion employing wild type PmlABCDEF.



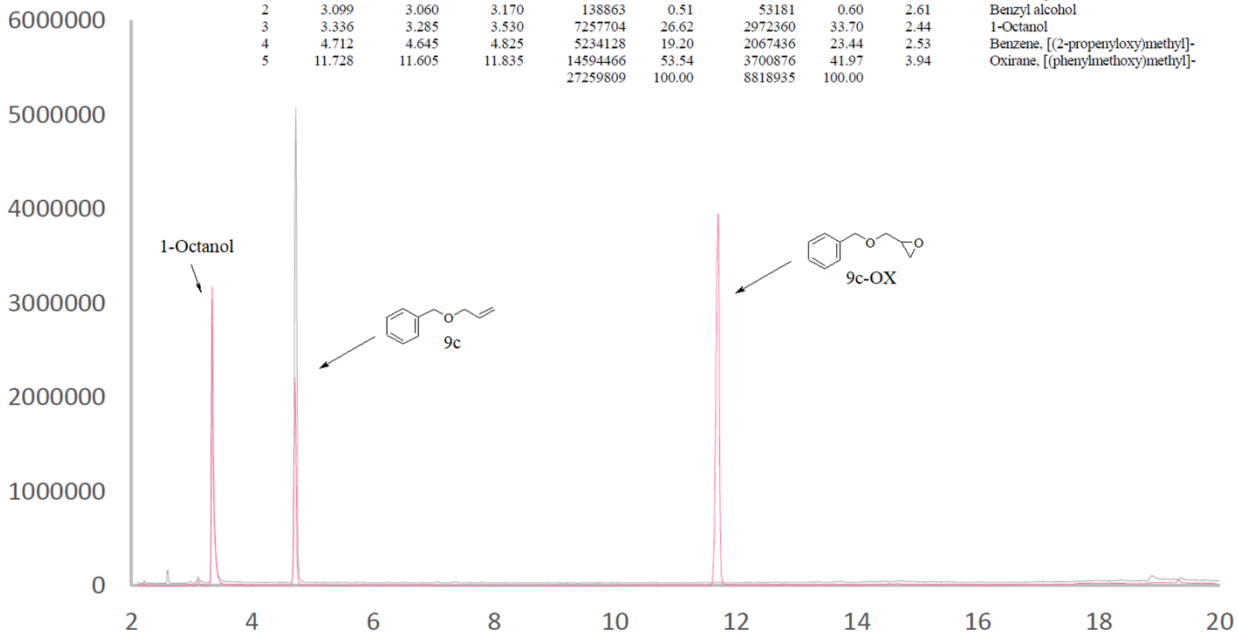
GC-MS analysis of *cis*-β-methylstyrene (**7c**) conversion employing wild type PmlABCDEF.



GC-MS analysis of allyl phenyl ether (**8c**) conversion employing wild type PmlABCDEF.

Peak Report TIC

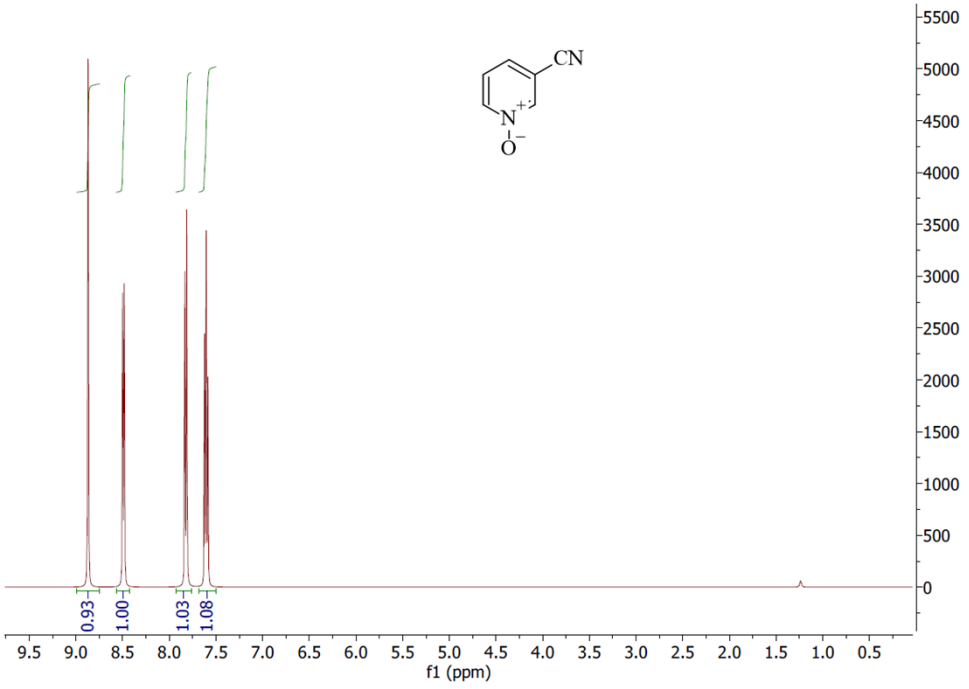
Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	2.210	2.180	2.240	34648	0.13	25082	0.28	1.38		1,3,5,7-Cyclooctatetraene
2	3.099	3.060	3.170	138863	0.51	53181	0.60	2.61		Benzyl alcohol
3	3.336	3.285	3.530	7257704	26.62	2972360	33.70	2.44		1-Octanol
4	4.712	4.645	4.825	5234128	19.20	2067436	23.44	2.53		Benzene, [(2-propenyloxy)methyl]-
5	11.728	11.605	11.835	14594466	53.54	3700876	41.97	3.94		Oxirane, [(phenylmethoxy)methyl]-
				27259809	100.00	8818935	100.00			

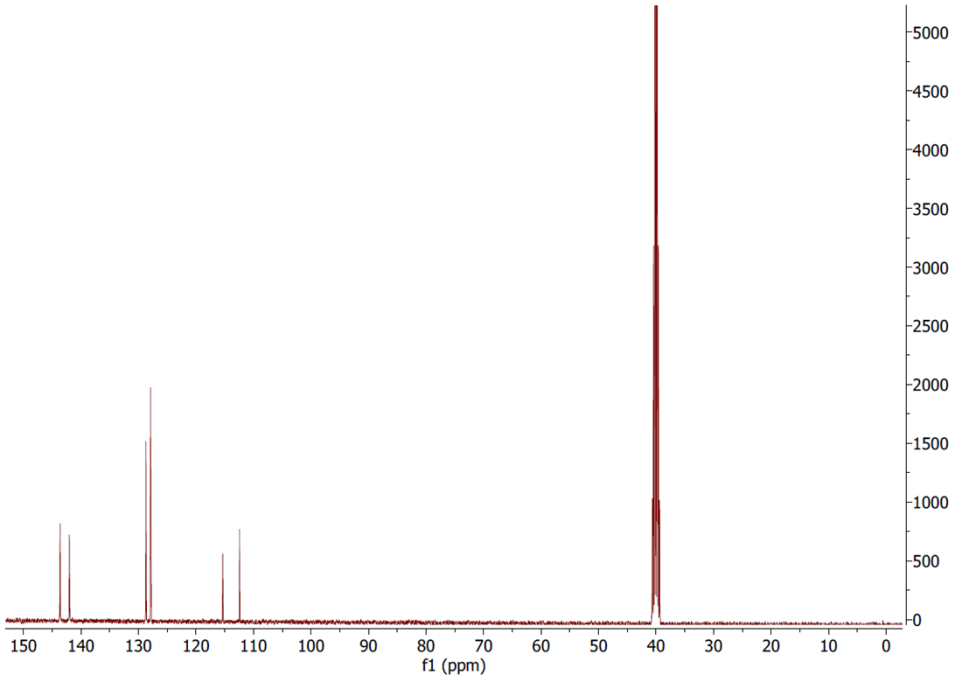


GC-MS analysis of allyl benzyl ether (**9c**) conversion employing wild type PmlABCDEF.

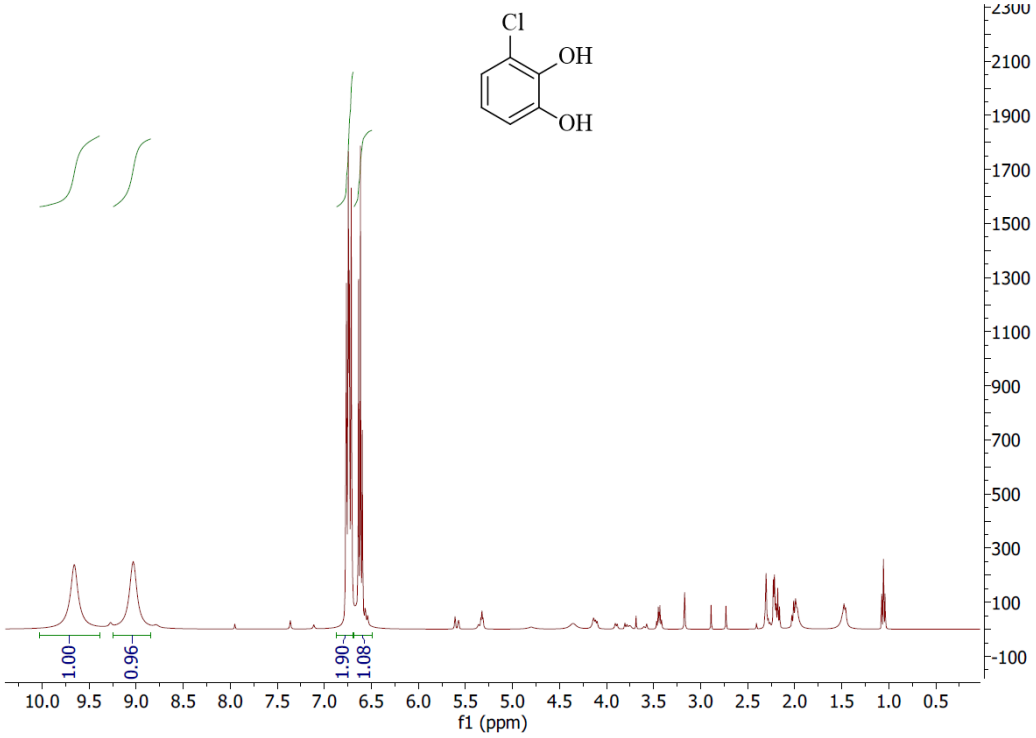
NMR spectra of synthesized compounds

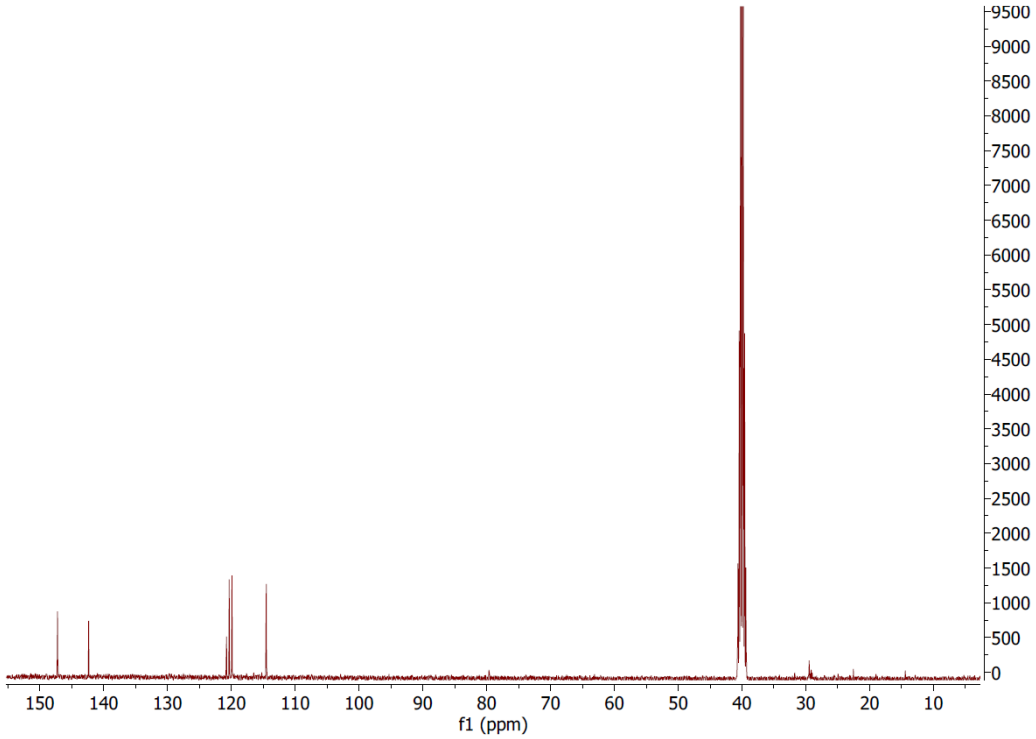
3-Cyanopyridine-1-oxide (**9a-1-NO**) was prepared according to the general biocatalysis procedure. The crude product was purified by flash chromatography on silica gel ($CHCl_3$: CH_3OH; 5 : 1) to give a white solid. ^1H NMR (400 MHz, DMSO-d6): δ = 7.60 (t, *J* = 7.3 Hz, 1H, CH), 7.82 (d, *J* = 7.9 Hz, 1H, CH), 8.49 (d, *J* = 7.8 Hz, 1H, CH), 8.87 (d, *J* = 1.7 Hz, 1H, CH). ^{13}C NMR (101 MHz, DMSO-d6): δ = 112.43, 115.35, 127.86, 128.7, 142.01, 143.64.



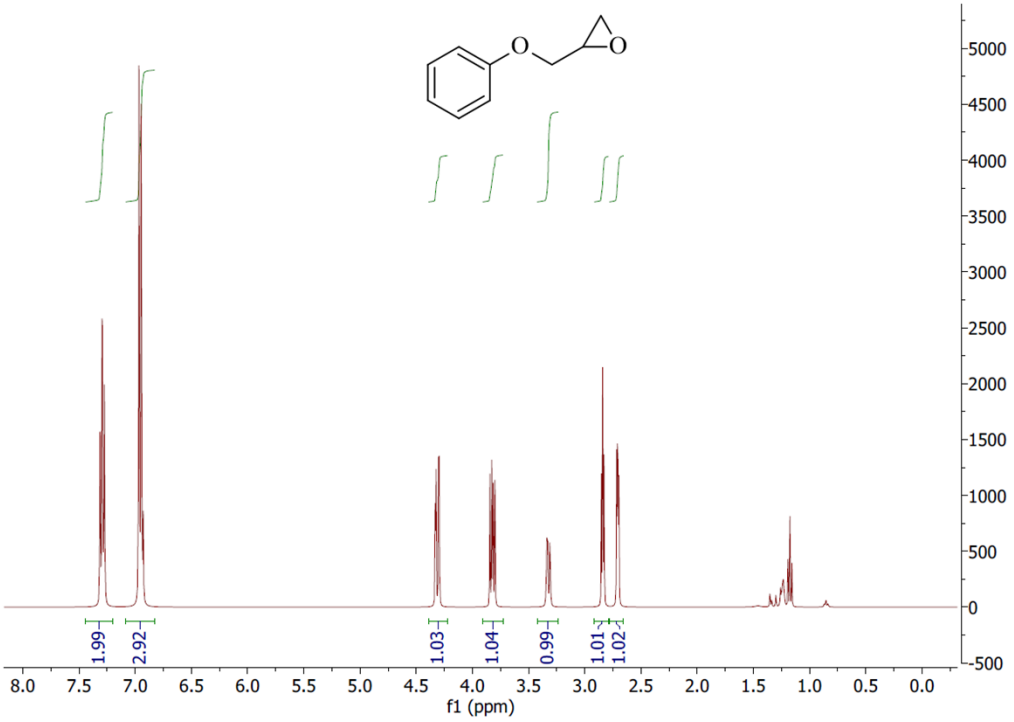


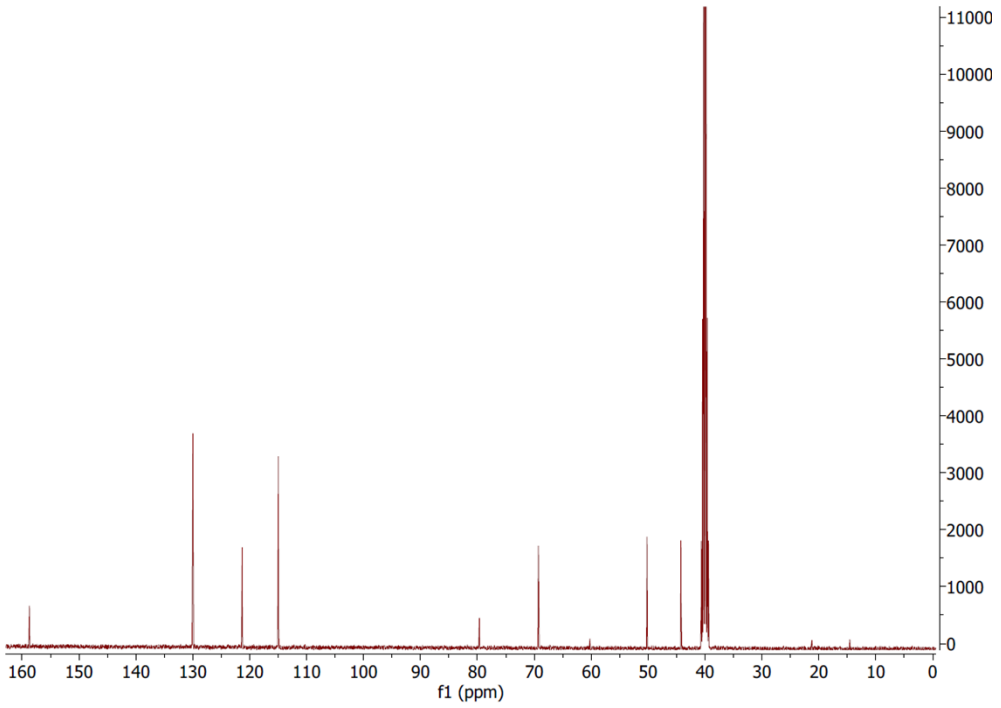
3-Chlorocatechol (**1e-OH**) was prepared according to the general biocatalysis procedure. The crude product was purified by flash chromatography on silica gel ($CHCl_3$: CH_3OH; 5 : 1) to give a brown solid. ^1H NMR (400 MHz, DMSO-d6): δ = 6.57 – 6.66 (m, 1H, CH), 6.69 – 6.81 (m, 2H, CH), 9.03 (br. s, 1H, OH), 9.66 (br. s, 1H, OH). ^{13}C NMR (101 MHz, DMSO-d6): δ = 114.52, 119.90, 120.33, 120.75, 142.35, 147.22.



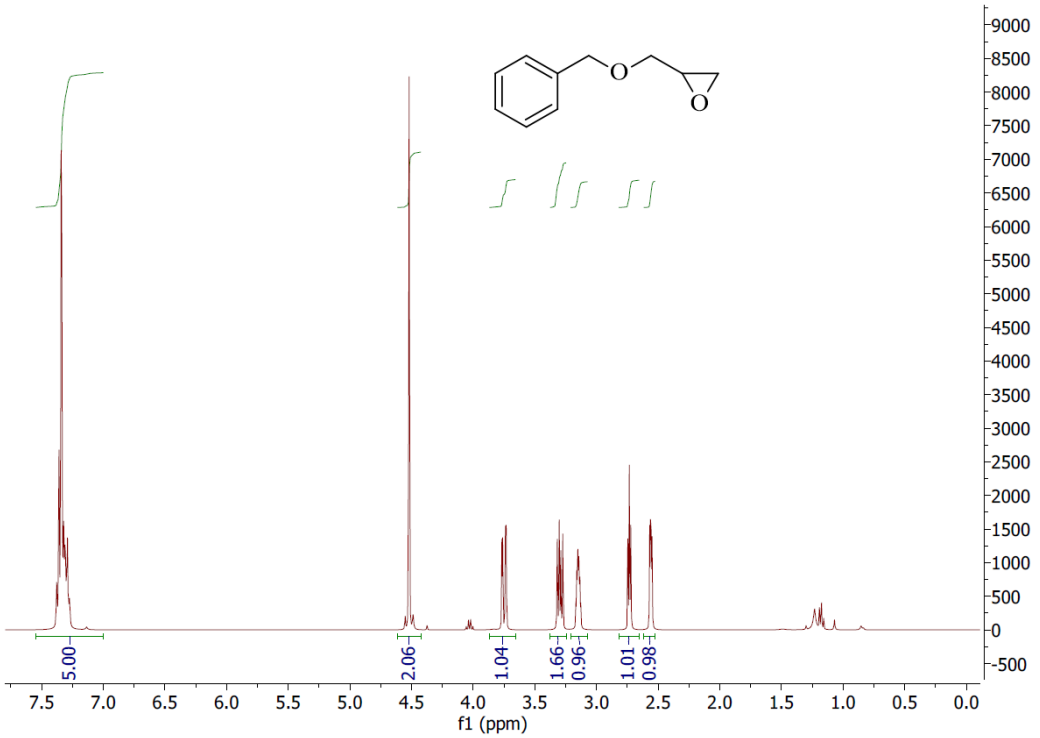


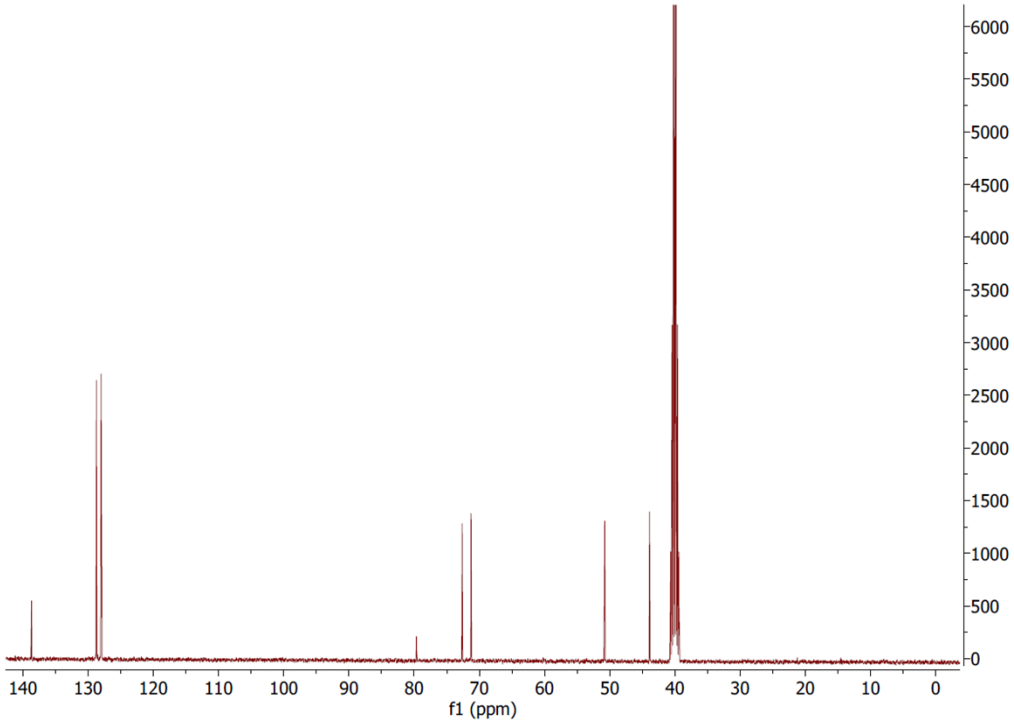
Phenyl glycidyl ether (**8c-OX**) was prepared according to the general biocatalysis procedure. The crude product was purified by flash chromatography on silica gel (n-hexane : EtOAc; 9 : 1) to give a colourless oily liquid. ^{1}H NMR (400 MHz, DMSO-d6): δ = 2.71 (dd, *J* = 5.1, 2.7 Hz, 1H, CH), 2.84 (dd, *J* = 5.1, 4.2 Hz, 1H, CH), 3.24 – 3.42 (m, 1H, CH), 3.82 (dd, *J* = 11.3, 6.5 Hz, 1H, CH), 4.31 (dd, *J* = 11.3, 2.7 Hz, 1H, CH), 7.08 – 6.83 (m, 3H, CH), 7.44 – 7.20 (m, 2H, CH). ^{13}C NMR (101 MHz, DMSO-d6): δ = 44.24, 50.19, 69.28, 114.96, 121.31, 129.98, 158.70.





Benzyl glycidyl ether (**9c-OX**) was prepared according to the general biocatalysis procedure. The crude product was purified by flash chromatography on silica gel (n-hexane : EtOAc; 9 : 1) to give a colourless oily liquid. ^{1}H NMR (400 MHz, DMSO-d6): δ = 2.56 (dd, *J* = 5.2, 2.7 Hz, 1H, CH), 2.73 (t, *J* = 4.7 Hz, 1H, CH), 3.07 – 3.21 (m, 1H, CH), 3.30 (dd, *J* = 11.5, 6.4 Hz, 1H, CH), 3.75 (dd, *J* = 11.5, 2.7 Hz, 1H, CH), 4.52 (s, 2H, CH), 7.26 – 7.39 (m, 5H, CH). ^{13}C NMR (101 MHz, DMSO-d6): δ = 43.88, 50.76, 71.27, 72.62, 127.95, 128.00, 128.73, 138.68.





TLC analysis of regioselective *N*-oxide synthesis

End products of the oxygenation reactions i) using *m*CPBA ii) WT Pml biocatalysis iii) A113G Pml biocatalysis were visualized on TLC for a better presentation. The exact structures of analysed compounds have been described in our previous study.

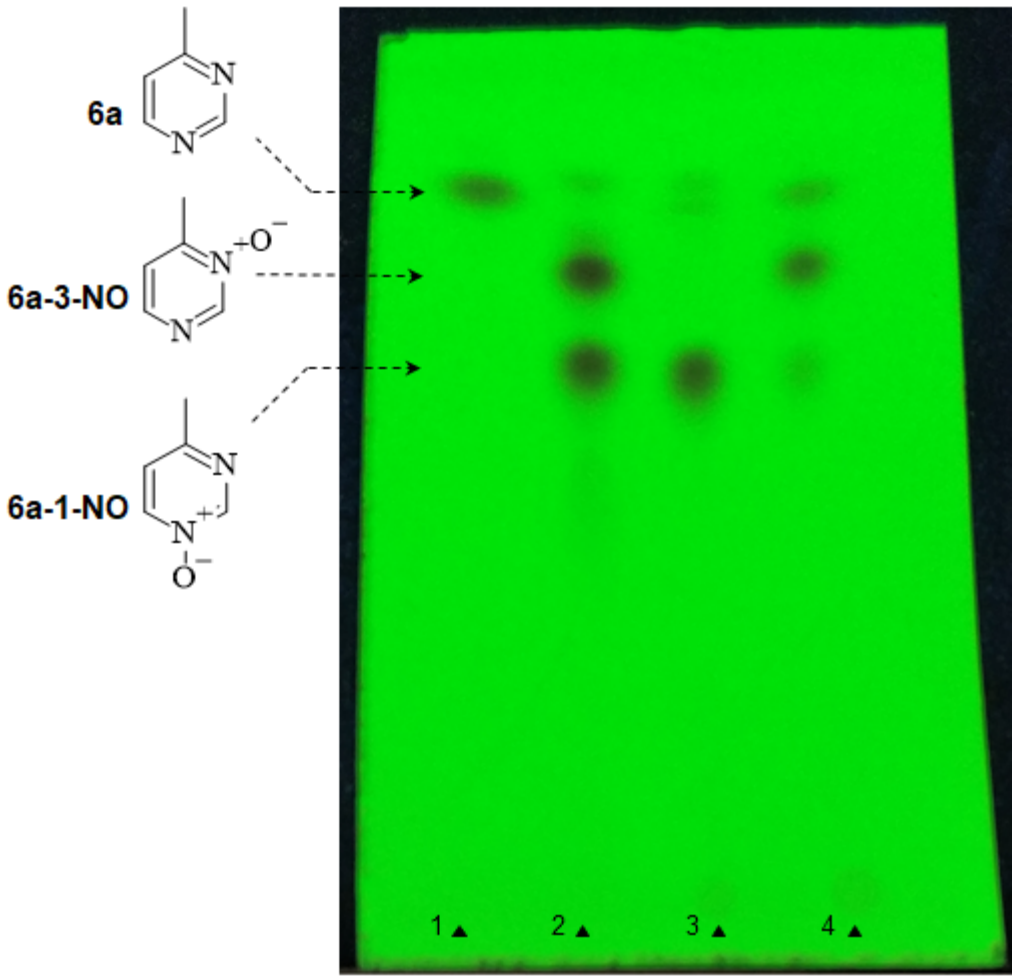


Fig. S1 TLC analysis of reaction mixtures for 4-methylpyrimidine (**6a**). The analysis was performed applying TLC Silica gel 60 F_{254} aluminium sheets from Merck, eluent system $CHCl_3$-MeOH (5:1). Lane 1 indicates control (starting compound), lane 2 – oxidation with *m*CPBA, lane 3 – biocatalysis with wild type PmlABCDEF, lane 4 – biocatalysis with PmlD A113G variant of PmlABCDEF.

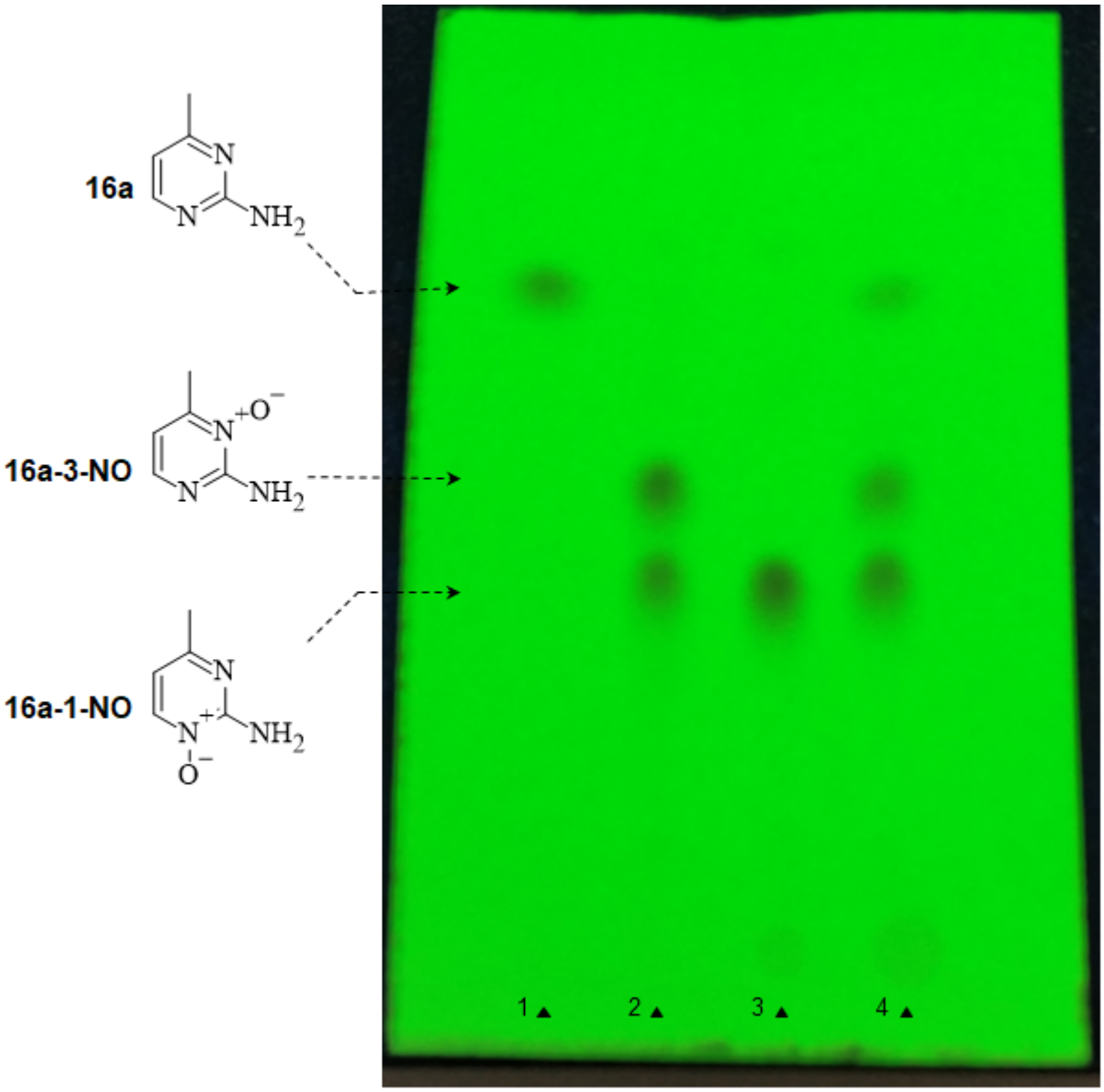


Fig. S2 TLC analysis of reaction mixtures for 2-amino-4-methylpyrimidine (**16a**). The analysis was performed applying TLC Silica gel 60 F_{254} aluminium sheets from Merck, eluent system $CHCl_3$-MeOH (5:1). Lane 1 indicates control (starting compound), lane 2 – oxidation with *m*CPBA, lane 3 – biocatalysis with wild type PmlABCDEF, lane 4 – biocatalysis with PmlD A113G variant of PmlABCDEF.

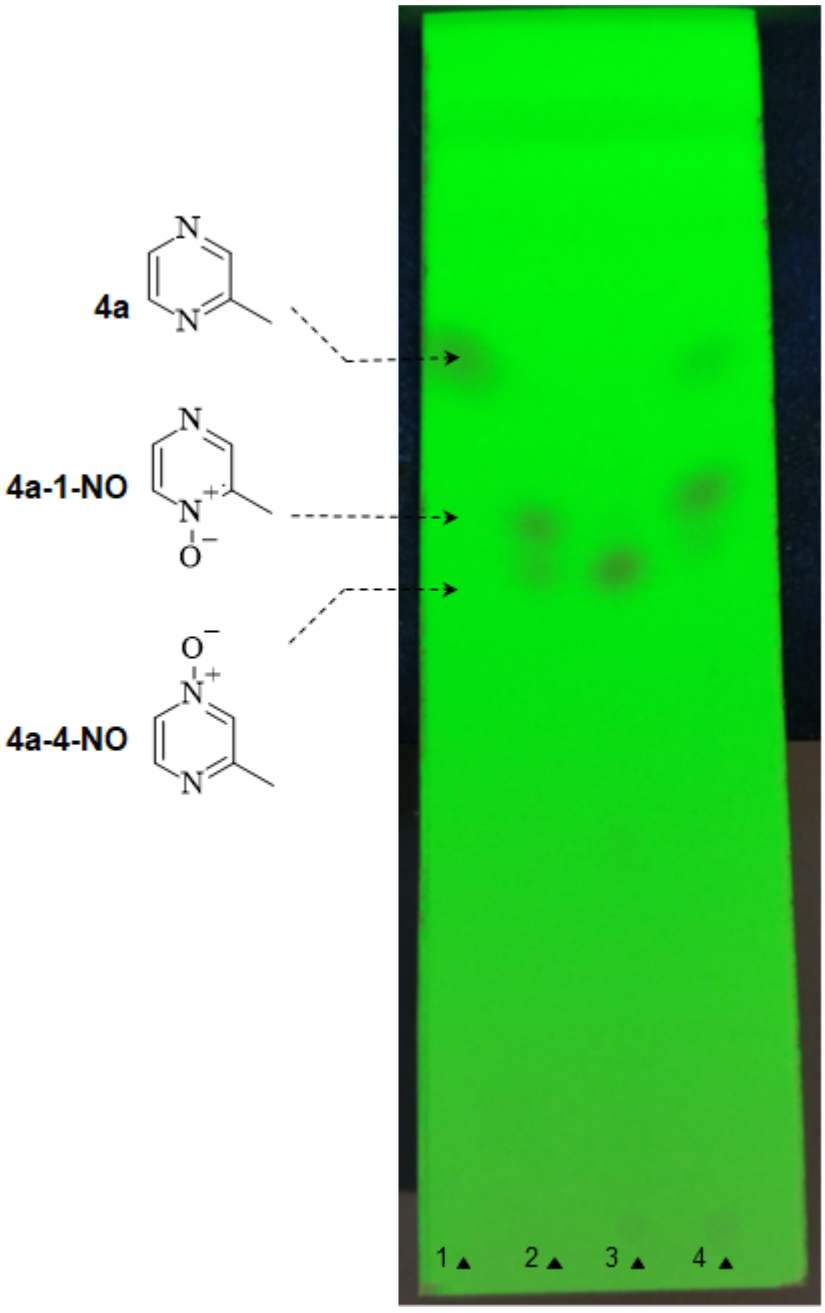


Fig. S3 TLC analysis of reaction mixtures for 2-methylpyrazine (**4a**). The analysis was performed applying TLC Silica gel 60 F_{254} aluminium sheets from Merck, eluent system $CHCl_3$-MeOH (9:1). Lane 1 indicates control (starting compound), lane 2 – oxidation with *m*CPBA, lane 3 – biocatalysis with wild type PmlABCDEF, lane 4 – biocatalysis with PmlD A113G variant of PmlABCDEF.