Streptavidin-hosted Organocatalytic Aldol Condensation

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Supporting Information

Table of Contents

1 General Information

Reactions were performed in oven dried glassware without precautions to exclude air. Reaction temperatures are stated as heating device temperature (e.g. oil bath, shaker, etc.), if not stated otherwise. Concentrations under reduced pressure were performed by rotary evaporation at 40°C at the appropriated pressure, unless otherwise noted. Deionized water was obtained by an *Elga PURELAB Option* system (15 MΩ·cm). Analytical and preparative Thin Layer Chromatography (TLC) was carried out with silica gel 60 F254 aluminium sheets from *Merck*. Detection was carried out using UV light (λ = 254 nm and 366 nm), followed by immersion in permanganate staining solution with subsequent development via careful heating with a heat gun. Flash column chromatography was performed using silica gel (pore size 60 Å, 0.040-0.063 mm).

para-Nitro benzaldehyde (**2**) for aldol reactions with **Sav** was obtained commercially (Sigma-Aldrich) and if necessary purified by washes with sodium bicarbonate pH 8.3, subsequent drying with magnesium sulphate and stored under inert atmosphere at 4 °C. All other solvents and reagents were obtained from commercial sources and used as received.

Sav (Streptavidin *Streptomyces avidinii* recombinant, tetramer, $M_w \approx 52$ kDa, Streptavidin with amino acids 13-139) was obtained commercially from *ProSpec* (PRO-791) as lyophilized powder in 10 mM KP_i pH 6.5 and stored at −23 °C upon receipt until further use. According to the supplier **Sav** has the following amino acid sequence:

```
MAEAGITGTWYNQLGSTFIVTAGADGALTGTYESAVGNAESRYVLTG
RYDSAPATDGSGTALGWTVAWKNNYRNAHSATTWSGQYVGGAEARIN
TQWLLTSGTTEANAWKSTLVGHDTFTKVKPSAAS
```

The plasmid for **T-rSav** (encoding for a Streptavidin *Streptomyces avidinii* recombinant, tetramer, $M_w \approx 50$ kDa, "reduced" Streptavidin with amino acids 16-133) was obtained as gift from Takeshi Sano (pTSA-13, Addgene plasmid # 17327, http://n2t.net/addgene:17327, RRID:Addgene_17327).[1] The gene encoding for **T-rSav** translates to the following amino acid sequence:

```
MGITGTWYNQLGSTFIVTAGADGALTGTYESAVGNAESRYVLTGRYD
SAPATDGSGTALGWTVAWKNNYRNAHSATTWSGQYVGGAEARINTQW
LLTSGTTEANAWKSTLVGHDTFTKV
```

A *VWR 3510* benchtop pH Meter connected to a *Jenway* micro pH electrode or a *VWR Universal* pH electrode were used for the pH adjustment of buffers and reaction mixtures employing either 1.0 M or 0.1 M sodium hydroxide solution or hydrochloric acid.

Shaking of the reactions (300 rpm) at 25 °C was achieved using a *VWR* thermoshaker *Mini shake lite* or a *VWR Incubating Orbital Shaker*.

^1H and ^{13}C NMR spectra were recorded in $CDCl_3$, methanol-d_4, DMSO-d_6, or D_2O on *Bruker Fourier 300*, *Ultrashield 400*, or *Ascend 500* instruments.

Size exclusion chromatography was performed using a ÄKTA Purifier workstation (GE Healthcare) or a Bio-Rad system with the respective column mentioned in the detailed procedure.

2 Experimental Details for the Preparation and Purification of T-rSav and mutants.

2.1 Expression and Purification

Tetrameric reduced streptavidin (**T-rSav**) and relative mutants were expressed using an *E. Coli* expression system with the following protocol. Plasmid pTSA-13 containing the desired **T-rSav** gene in a pET-3a vector was transformed into calcium competent BL21(DE3) pLysS cells and grown for 16 h on LB agar plates containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. A single colony from the plate was picked to inoculate a 15 mL MTP (per 1 L: 10 g tryptone, 10 g NaCl, 5 g yeast extract, 2.2 g Na_2HPO_4, 1 g KH_2PO_4, pH = 6.9) starter culture containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol, which was incubated at 37 °C and 180 rpm overnight. The culture was diluted to 40 mL with 20% glucose and then added to 1 L MTP medium containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol, yielding a final glucose concentration of 0.05%. The cultures were grown at 37 °C and 225 rpm to an OD_{600} of 1.0−1.2 and induced with IPTG at a final concentration of 1 mM. The culture was grown at 25 °C for 16 h and the cell pellet was harvested after centrifugation at 4000 rpm at 4 °C for 25 min and stored at −20 °C.

The pellet was subjected to a freeze-thaw cycle, resuspended in 25 mL of lysis buffer (50 mM Tris, 100 mM NaCl, 1 mM PMSF, pH 8.0) and lysed by sonication (7 min, 5 s on, 10 s off). The insoluble fraction was isolated by centrifugation at 15000 rpm for 25 min at 4 °C. The supernatant was discarded and the insoluble fraction was washed with wash buffer 1 (4× resuspension in 50 mM Tris, 110 mM EDTA, 1.5 M NaCl, 1 mM PMSF, 0.1% Triton X-100, pH 8.0 and pellet re-isolation by centrifugation at 11000 rpm and 4 °C) and wash buffer 2 (4× resuspension in 50 mM Tris, 110 mM EDTA, 1.5 M NaCl, 1 mM PMSF, pH 8.0 and pellet re-isolation by centrifugation at 11000 rpm and 4 °C). The insoluble fraction was resuspended in denaturing buffer 1(5 mL / g pellet, 6 M GdnHCl, 50 mM Tris-HCl, pH 1.5) and incubated at 37 °C and 180 rpm for 16 h. The insoluble fraction was removed by centrifugation at 15000 rpm and 4 °C. The supernatant was diluted to 200 mL with denaturing buffer 2 (6 M GdnHCl, 50 mM Tris-HCl, pH 6.5) and dialysed against 3 L of 6 M GdnHCl, 50 mM Tris-HCl, pH 6.5 for 3 h at room temperature. The dialysis bag was then placed into fresh 3 M GdnHCl, 50 mM Tris-HCl, pH 6.5 (denaturing buffer 2 was reused up to 5 times). **T-rSav** was refolded by gradient dialysis, pumping in refolding buffer (0.5 mg/L catalyst **1**, 10 mM KP_i, pH 7.0) at 4 mL/min, constant stirring and removal of the mixture at 4 mL/min for 48 h at room temperature. Towards the end of this process a varying amount of precipitation was observed. The precipitate was removed via centrifugation at 15000 rpm at 4 °C and the supernatant was concentrated to 20 mL by Amicon ultra centrifugation using a 3.5 kDa cut-off. The concentrated solution was transferred into a centrifugal concentrator with a 10 kDa cut-off and the buffer was exchanged five times by concentration to 2.5 mL and refilling to 20 mL (10 mM KP_i, pH 7.0). The protein solution was finally concentrated to obtain a protein concentration of

2 mg/mL as determined by nanodrop measurement at 210 nm. This was used for catalysis of the aldol reaction without further purification. A sample of the solution was loaded on SDS-PAGE to check the purity of the protein (15% w/v).

2.2 Site-directed mutagenesis

The mutations K121 or S112 were introduced by site-directed mutagenesis PCR using PrimeStar HS DNA polymerase (Takara) and the accompanying buffers, dNTPs and primers mentioned in Table S1 below. Due to the high GC content of the region of interest a variety of methods and temperatures had to be screened, as primer insertions were observed, especially for mutations at K121. Hence a 50 µL PCR was prepared according to the instructions and the reaction mixture distributed equally (12.5 µL) over 4 PCR tubes. These were then subjected to the following conditions, using a gradient to achieve a different annealing temperature for each tube:

Method 1

Initial denaturing:	4 min, 95 °C
33 cycles:	10 s, 98 °C
	5 s, 58/60/62/64 °C
	5 min, 72 °C
Final extension:	10 min, 72 °C
Hold:	4 °C

Method 2

Initial denaturing:	4 min, 95 °C
15 cycles:	10 s, 98 °C
	5 s, 58/60/62/64 °C
	5 min, 72 °C
15 cycles:	10 s, 98 °C
	5 s, 61/63/65/67 °C
	5 min, 72 °C
Final extension:	10 min, 72 °C
Hold:	4 °C

Method 3

Initial denaturing:	4 min, 95 °C
3 cycles:	10 s, 98 °C
	5 s, 55/57/59/61 °C
	5 min, 72 °C
3 cycles:	10 s, 98 °C
	5 s, 58/60/62/64 °C
	5 min, 72 °C
30 cycles:	10 s, 98 °C
	5 s, 61/63/65/67 °C

	5 min, 72 °C
Final extension:	10 min, 72 °C
Hold:	4 °C

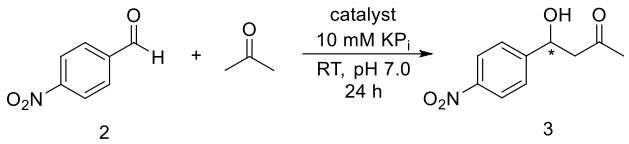
In case of the K121 mutations, Method 1 and 3 were also applied using 3% DMSO, if no positive results were obtained without DMS. The mutant constructs were confirmed by DNA sequencing (Eurofins, Genomics) using the T7 promoter primer (TAATACGACTCACTATAGG).

Table S1. List of primers used for the introduction of mutations in **T-rSav** at positions S112 and K121.

Mutation	**Primer (5' to 3')**
S112E	Forward GGCTGCTGACC**GAA**GGCACCACCGAGG Reverse CCTCGGTGGTGCC**TTC**GGTCAGCAGCC
K121A	Forward ACCGAGGCCAACGCCTGG**GCG**TCCACGCTGGTCGGC Reverse GGCGTTGGCCTCGGTGGTGCCGGA

3 Experimental Details for the Activity Screening of Catalysts 1 for the Aldol Reaction of Acetone and *p*-Nitro Benzaldehyde

3.1 ^{1}H NMR Based Screening for Yield Determination of Aldol Reaction of Acetone and *p*-Nitro Benzaldehyde

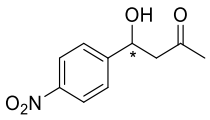


2 3

A stock solution of catalyst **1** (0.50 mg, 1.59 µmol) was prepared dissolving the catalyst in 1 mL of KPi (pH 7.0, 10 mM) into a 1.5 mL Eppendorf tube. A stock solution of *p*-nitro benzaldehyde (**2**, 20 mg, 132.34 µmol) was weighted and dissolved in 1 mL of **acetone**. Commercial Sav (0.58 mg, 1 mol%, 33 nmol) was weighted into a 1.5 mL Eppendorf tube and dissolved in 379.25 µL of KP_i (pH 7.0, 10 mM). An aliquot of 20.75 µL (1 mol%) of the catalyst stock solution was added to the Sav Eppendorf tube. Subsequently, an aliquot of 24.93 µL (1 eq.) of the *p*-nitro benzaldehyde stock solution was added to the Eppendorf tube. 75.07 µL of **acetone** were added to reach a final **acetone** volume of 100 µL. The mixture was shaken at 300 rpm at 25 °C for 24 hrs. The mixture was extracted with CH_2Cl_2 (500x3 µL) and the organic phase evaporated under reduced pressure. The crude of reaction was dissolved in $CDCl_3$ (620 µL) and subjected to ^{1}H NMR analysis.

4 Synthesis and ^{1}H-NMR Assignment of Aldol Reaction Product

4.1 Synthesis and ^{1}H-NMR assignment of 4-hydroxy-4-(4-nitrophenyl) butan-2-one (3)



3

4-hydroxy-4-(4-nitrophenyl) butan-2-one (**3**) was synthesised as previously reported.[2] The analytical data have been found to be in good agreement with the reported data.[2]

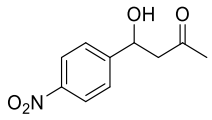
5 Chiral HPLC Data of Activity and Selectivity Screening

5.1 Screening Reactions

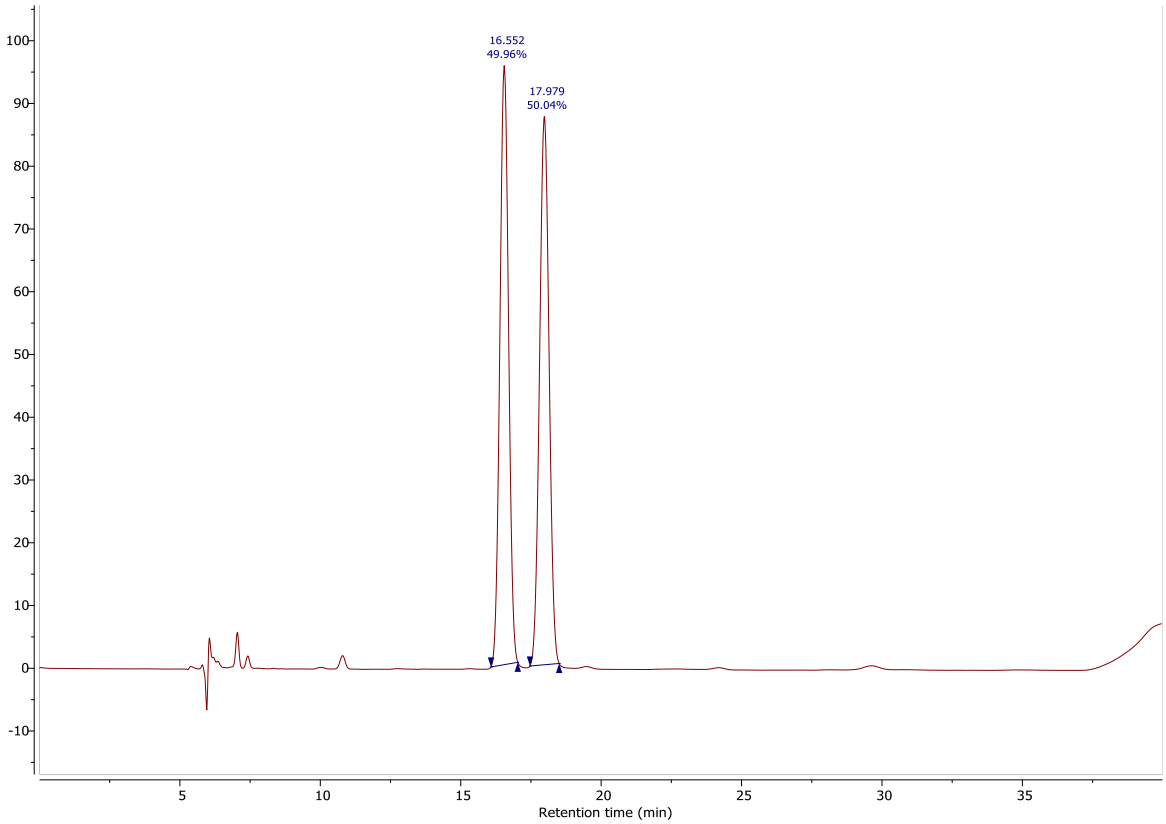
Analytical chiral HPLC analysis of product **3** was performed on an *Agilent Technologies 1260 Infinity Quaternary* LC system using a different columns. Slight variations on the retention time are due to change of the column guard throughout the measurements. A *Phenomenex Lux Amylose-1* column, 4.6 mm × 250 mm (0.5 mL/min, 25 °C, *n*-hexane/*iso*-propanol 75:25, 50 min), was employed. The peaks were assigned using signal at 280 nm.

5.1.1 Racemate 3

Racemic samples of **3** were obtained following a known procedure, using piperidine as catalyst.



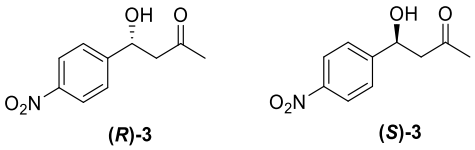
3



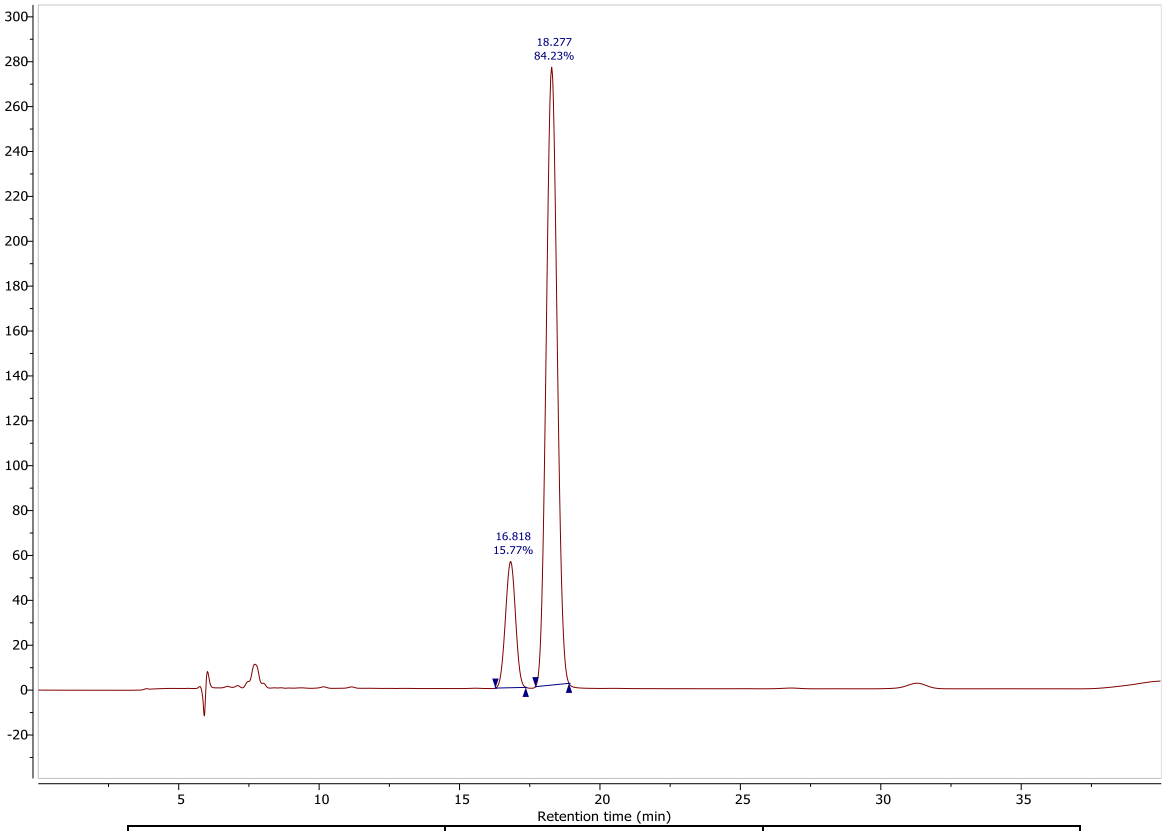
Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	16.5	50.0
2 (*S*-enantiomer)	18.0	50.0

5.1.2. L-proline

The absolute stereochemistry of **3** was assigned after running the sample obtained using L-Proline as catalyst.

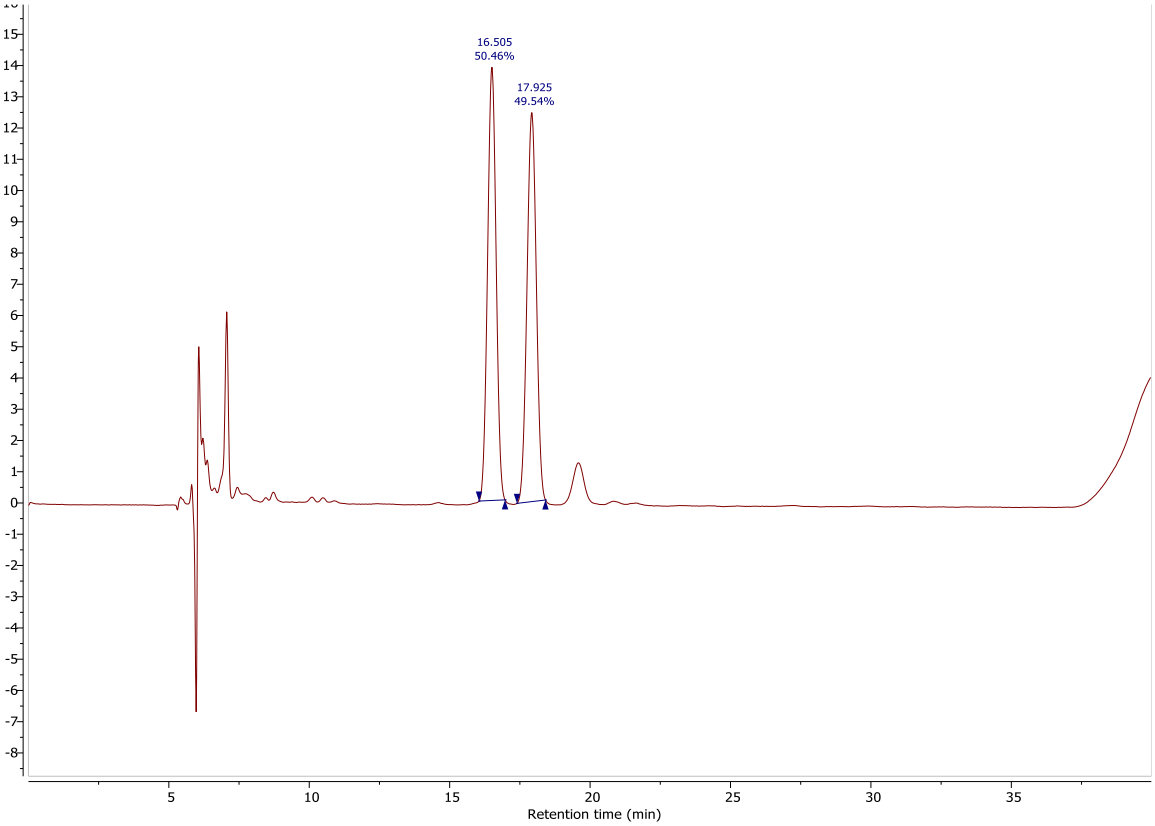


(*R*)-3 (*S*)-3



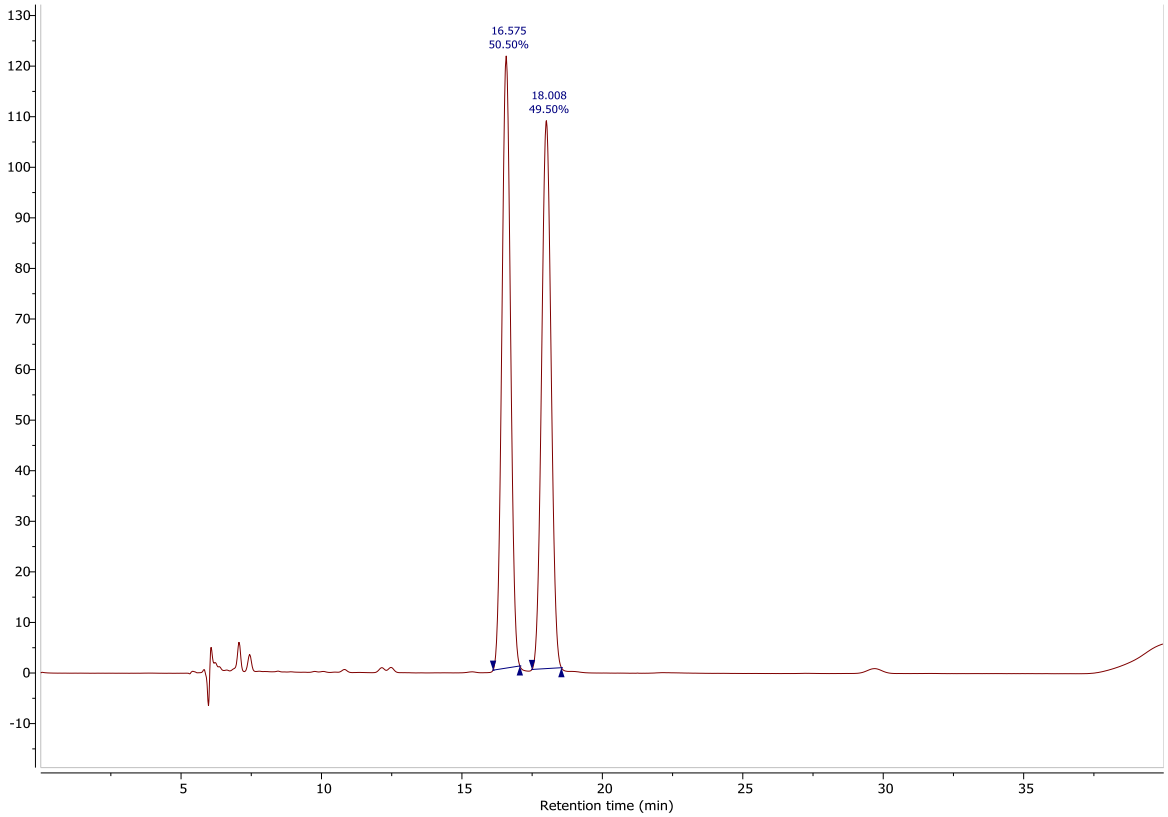
Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	16.8	15.7
2 (*S*-enantiomer)	18.3	84.3

5.1.3 Catalyst 1 (1 mol%)



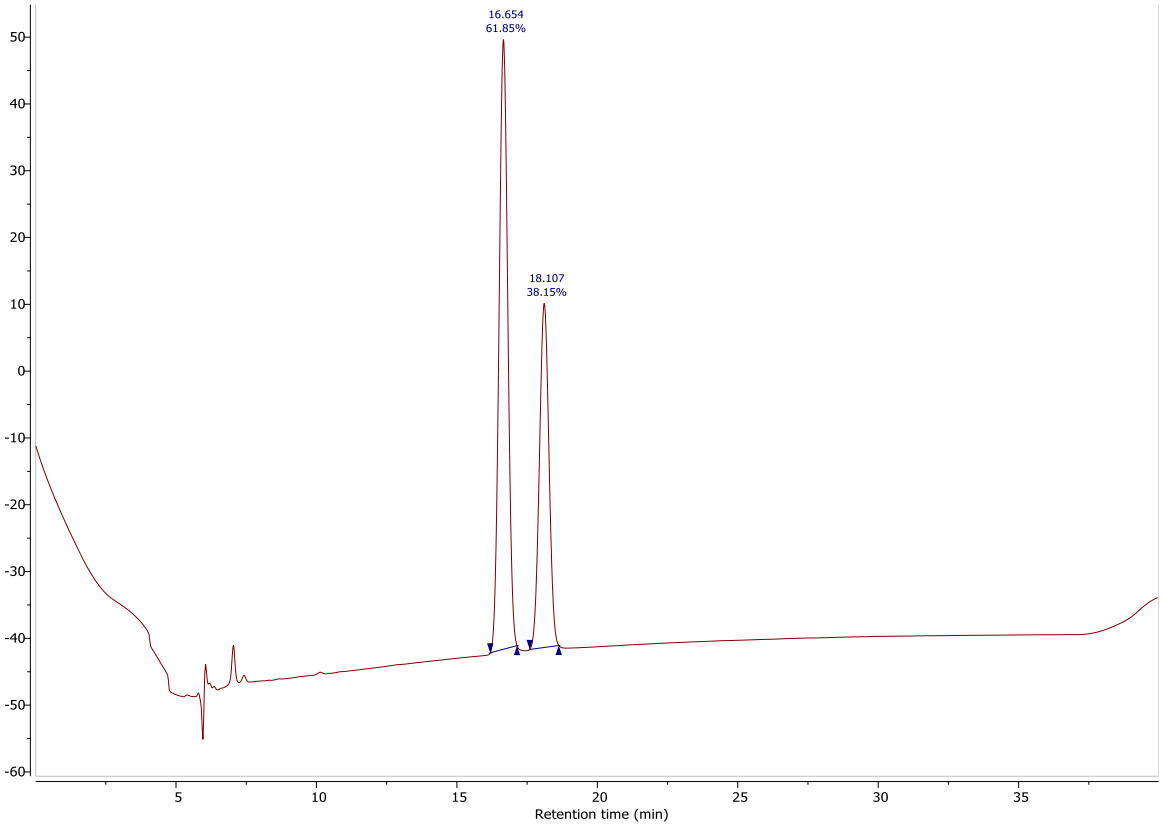
Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	16.5	50.5
2 (*S*-enantiomer)	17.9	49.5

5.1.4 Sav (1 mol%)



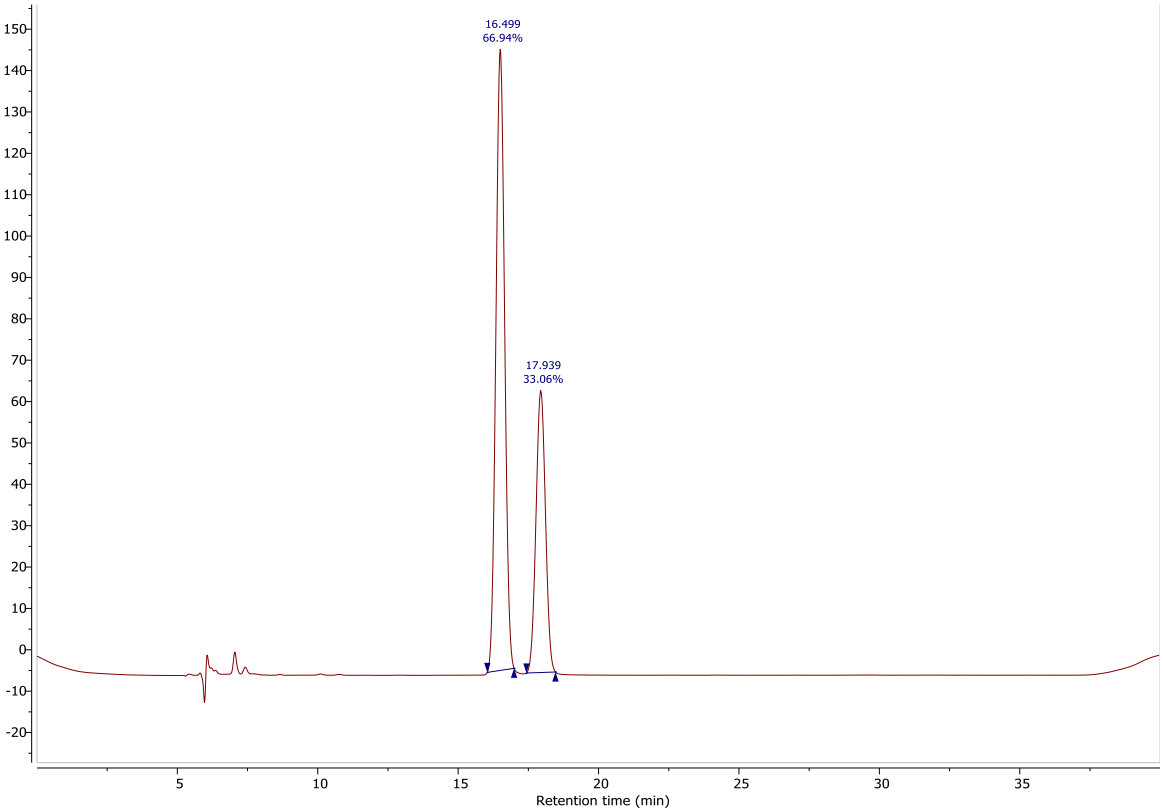
Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	16.5	50.5
2 (*S*-enantiomer)	18.0	49.5

5.1.5 Sav:1 (0.1 mol%)



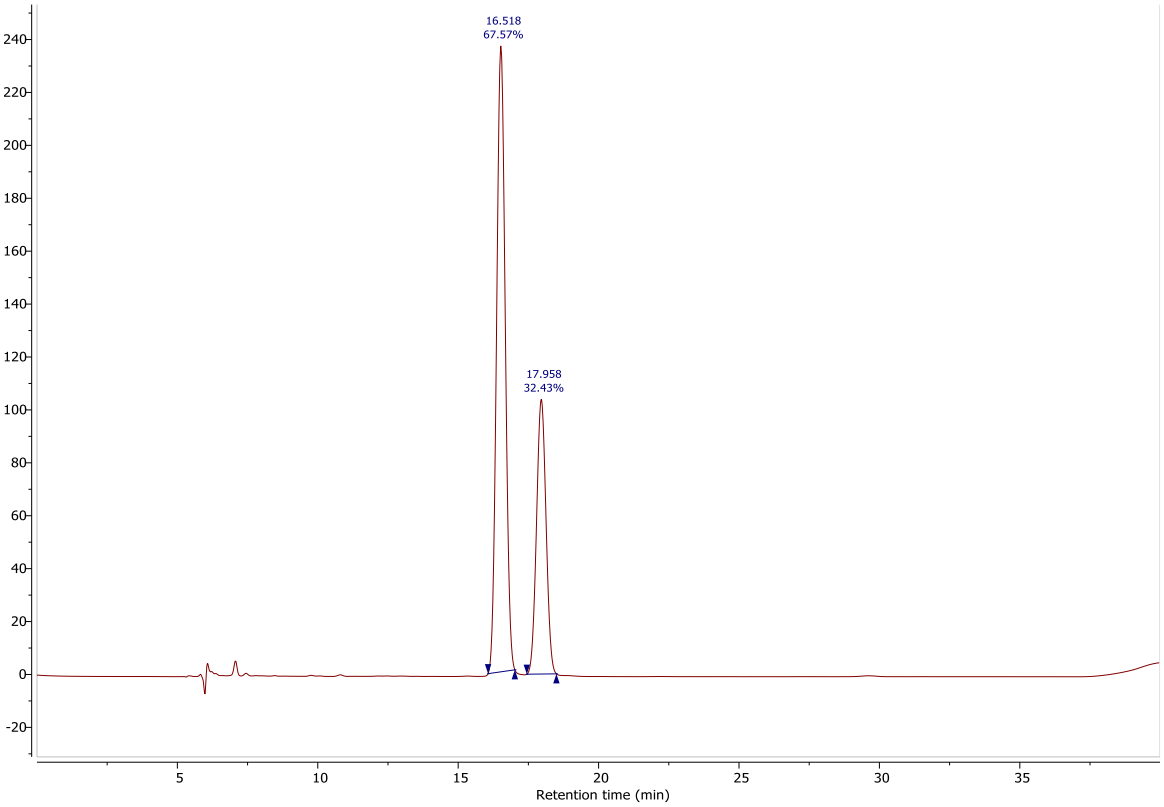
Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	16.6	61.9
2 (*S*-enantiomer)	18.1	38.1

5.1.6 Sav:1 (0.5 mol%)



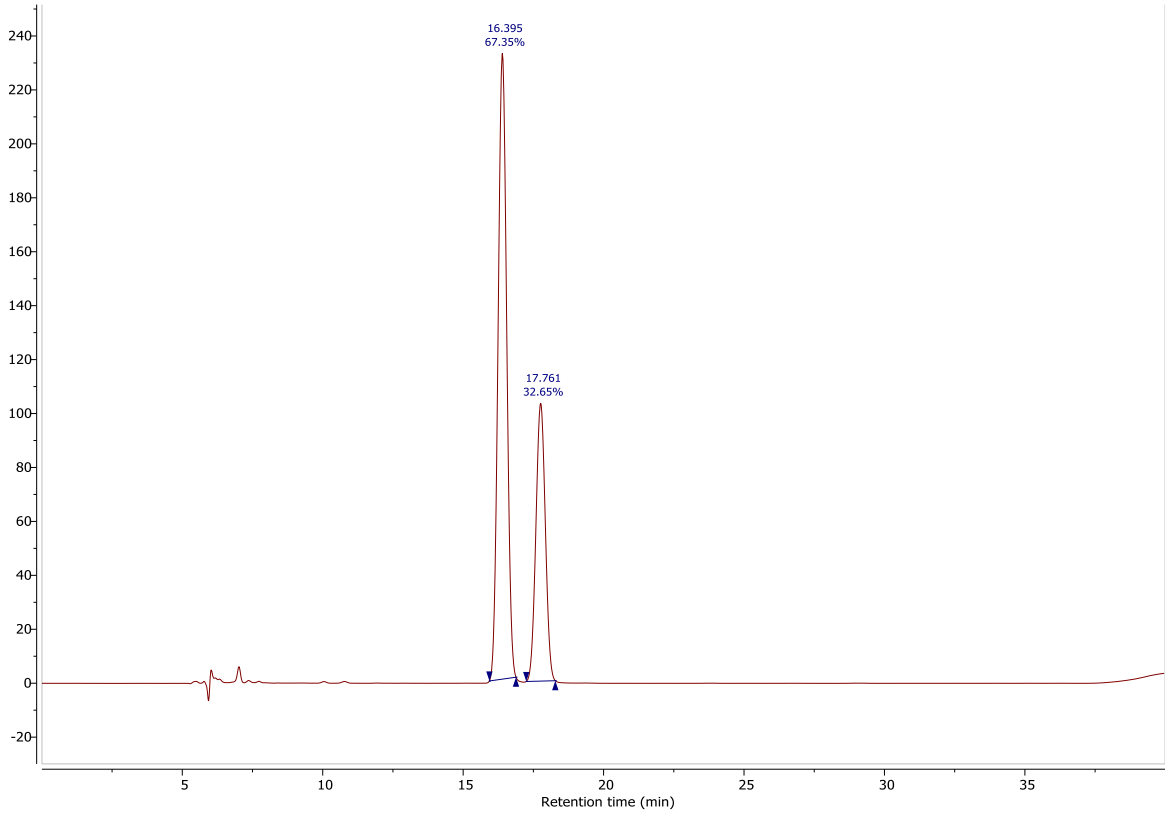
Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	16.5	67.0
2 (*S*-enantiomer)	17.9	33.0

5.1.7 Sav:1 (1 mol%)



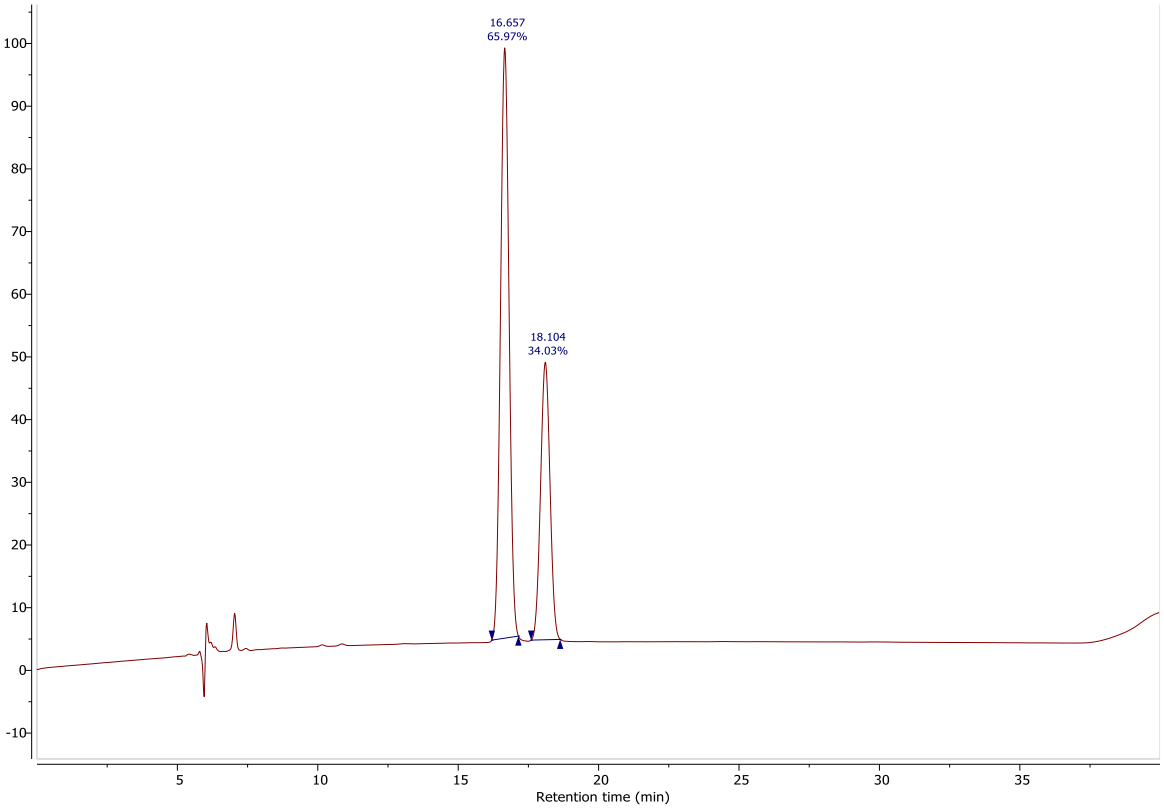
Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	16.5	67.6
2 (*S*-enantiomer)	17.9	32.4

5.1.8 Sav:1 (1 mol% + 1mol% TFA)



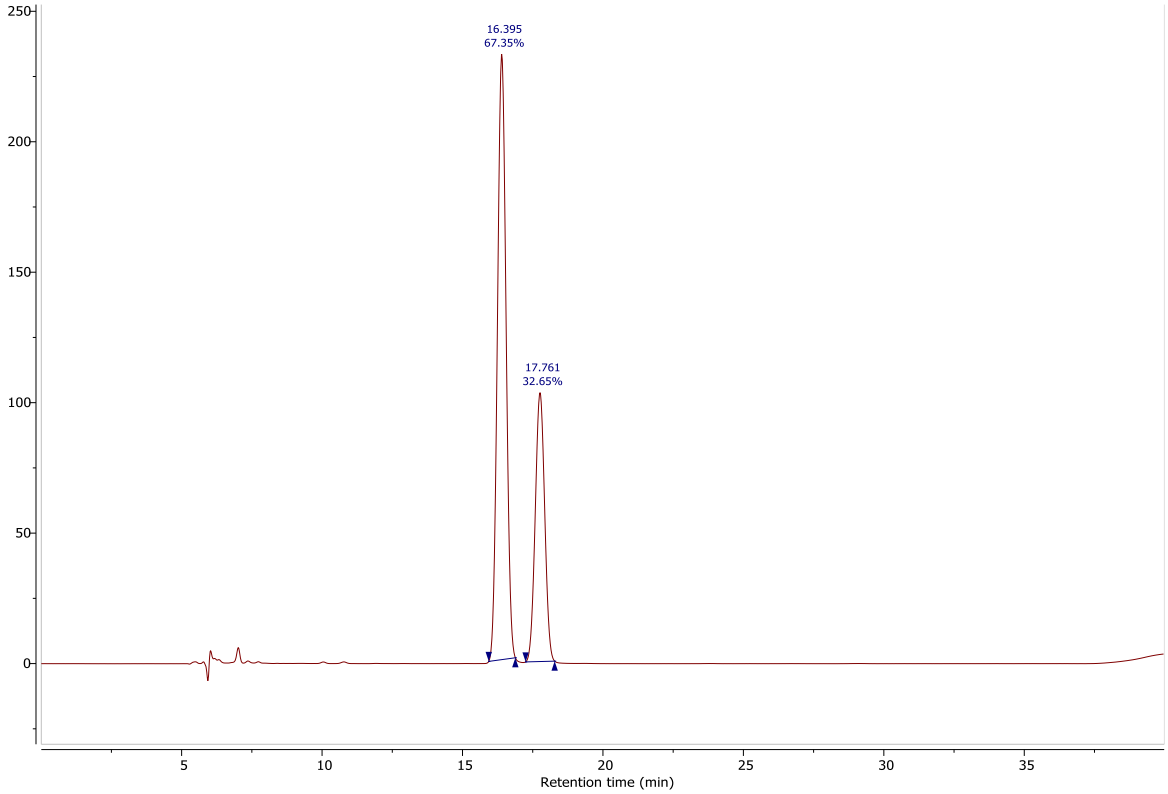
Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	16.4	67.4
2 (*S*-enantiomer)	17.8	32.6

5.1.9 Sav:1 (1 mol%) at 10 °C



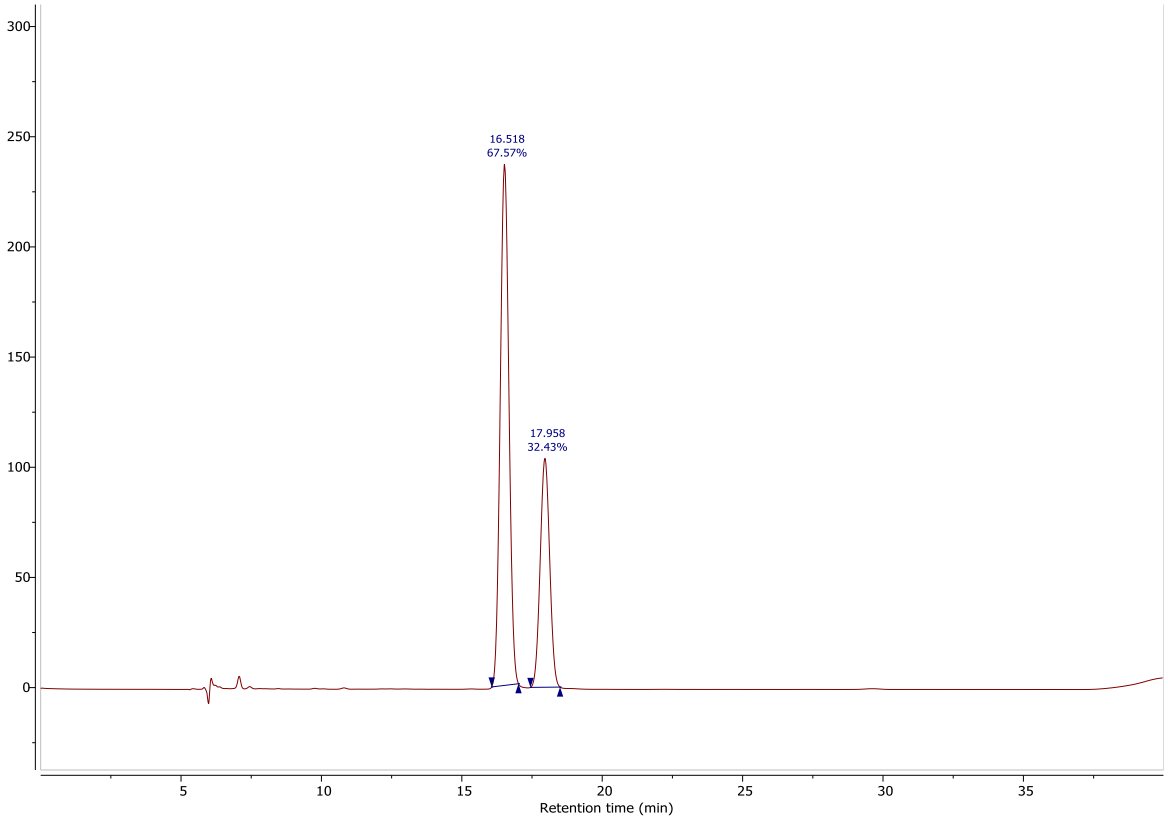
Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	16.6	66.0
2 (*S*-enantiomer)	18.1	34.0

5.1.10 Sav:1 (1 mol%) using 5 equivalent of acetone and 25% methanol



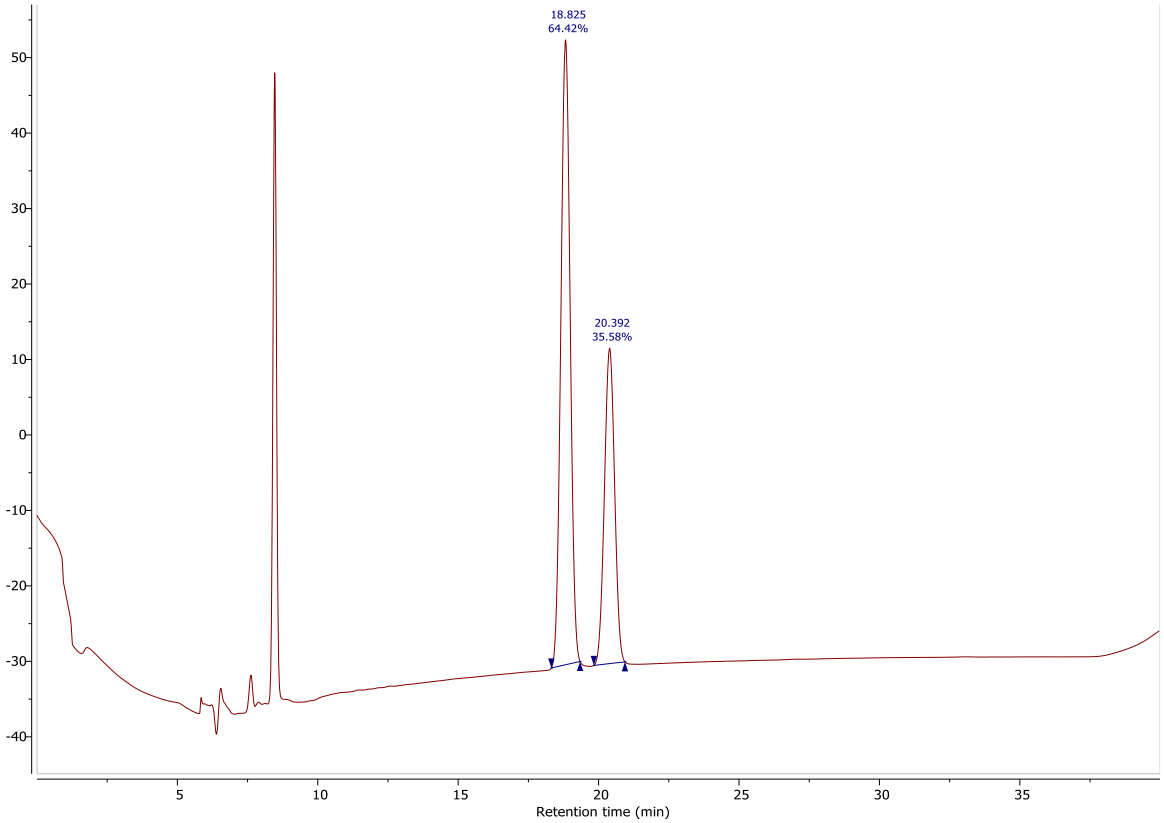
Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	16.4	67.4
2 (*S*-enantiomer)	17.8	32.6

5.1.11 Sav:1 (1 mol%) using 5 equivalent of acetone and 25% acetonitrile



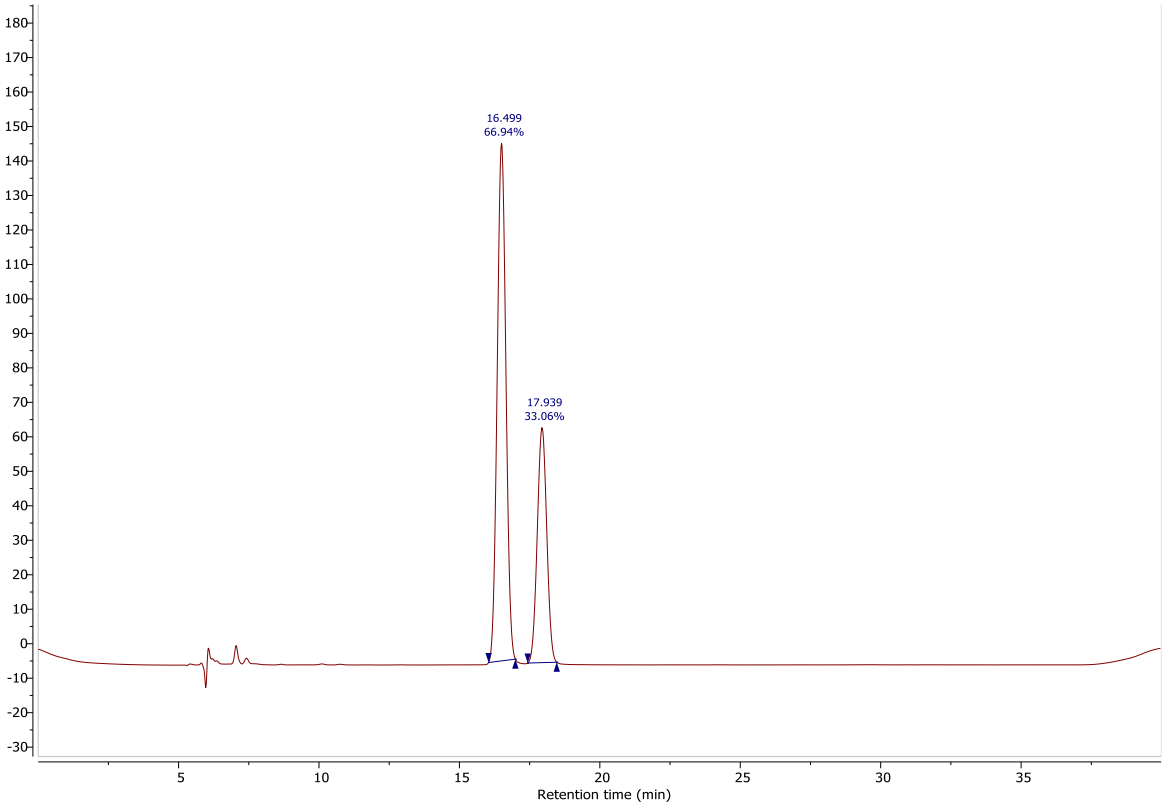
Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	16.5	67.6
2 (*S*-enantiomer)	17.9	32.4

5.1.12 Sav:1 (1 mol%) using 20 equivalent of acetone and 25% iso-propanol



Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	18.8	64.4
2 (*S*-enantiomer)	20.4	35.6

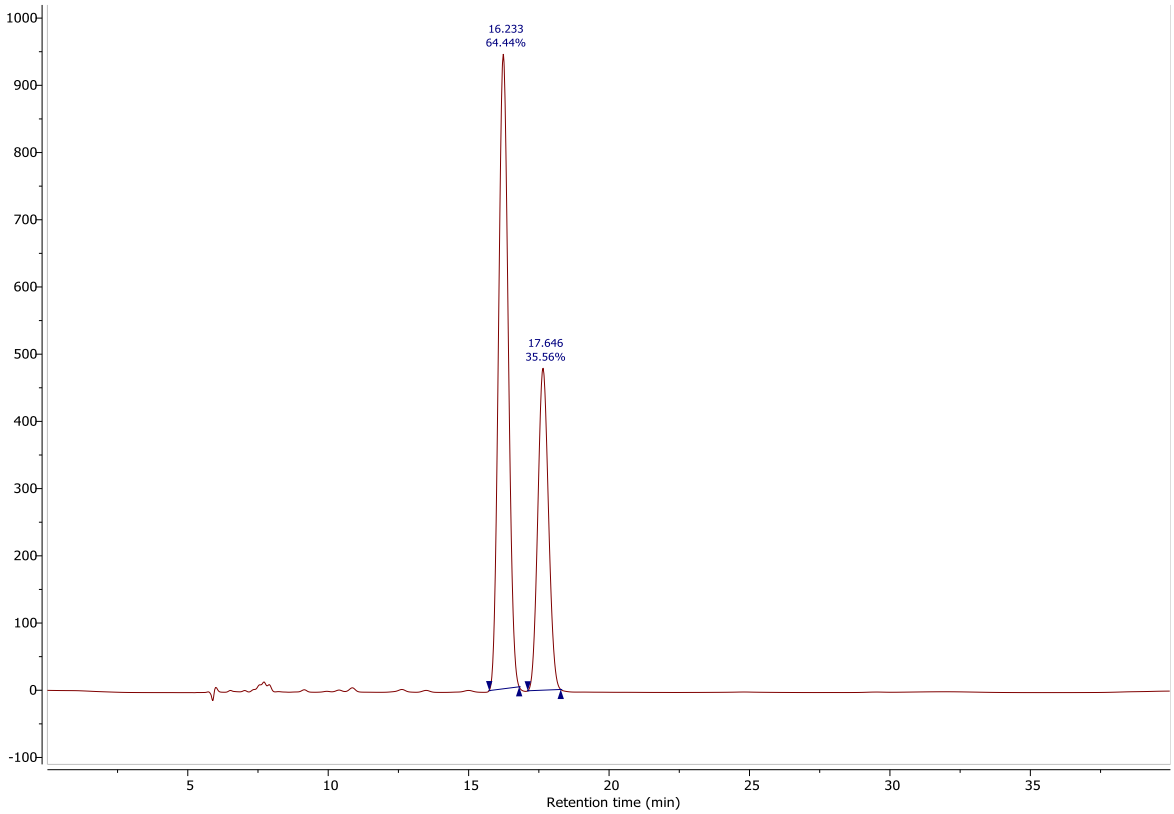
5.1.13 Sav:1 (1 mol%) using 50 equivalent of acetone and 25% iso-propanol



Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	16.4	66.9
2 (*S*-enantiomer)	17.9	33.1

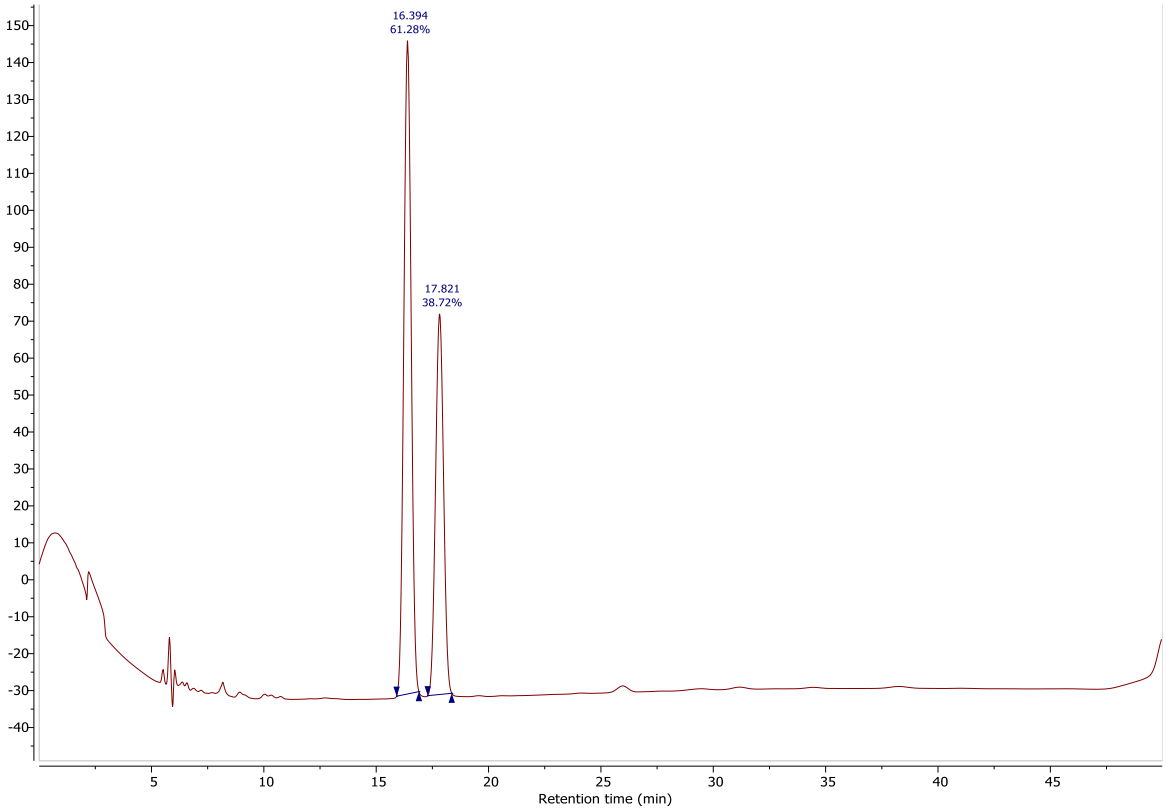
5.2 T-rSav and mutants:1 Reactions

5.2.1 Tr-Sav:1



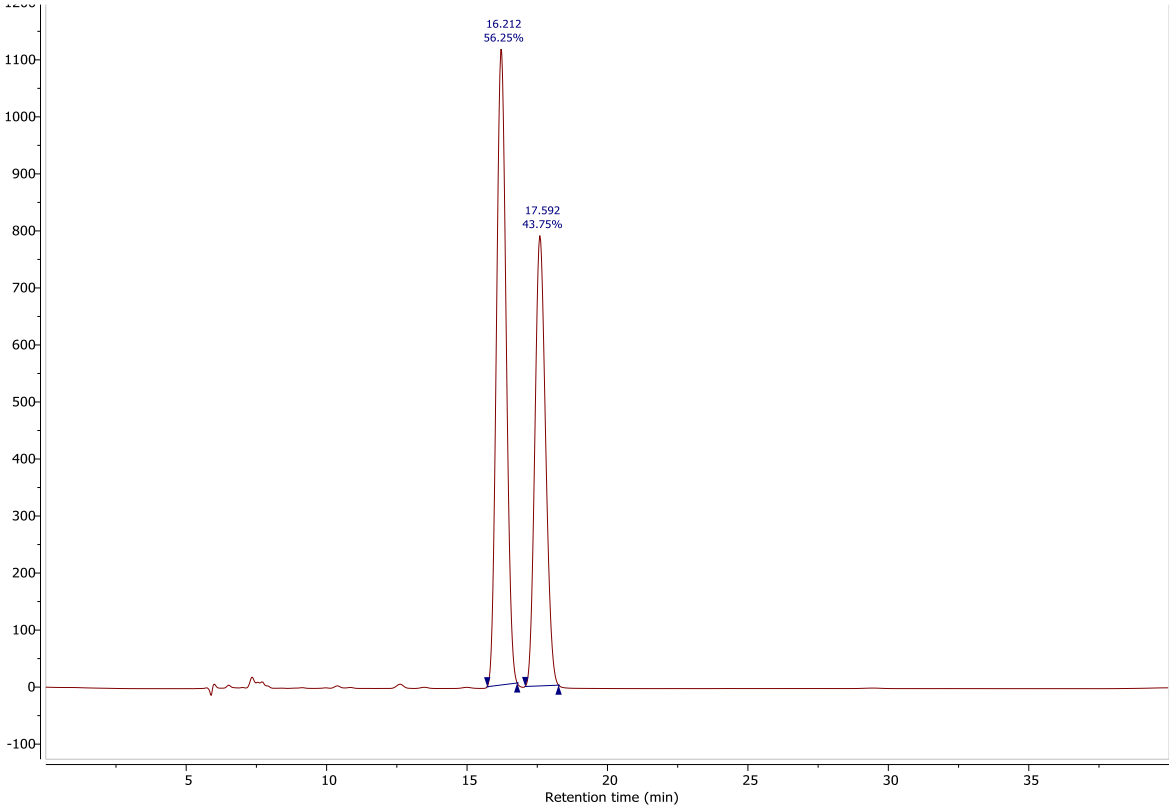
Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	16.2	64.4
2 (*S*-enantiomer)	17.6	35.6

5.2.2 S112E:1



Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	16.4	61.3
2 (*S*-enantiomer)	17.8	38.7

5.2.3 K121A:1

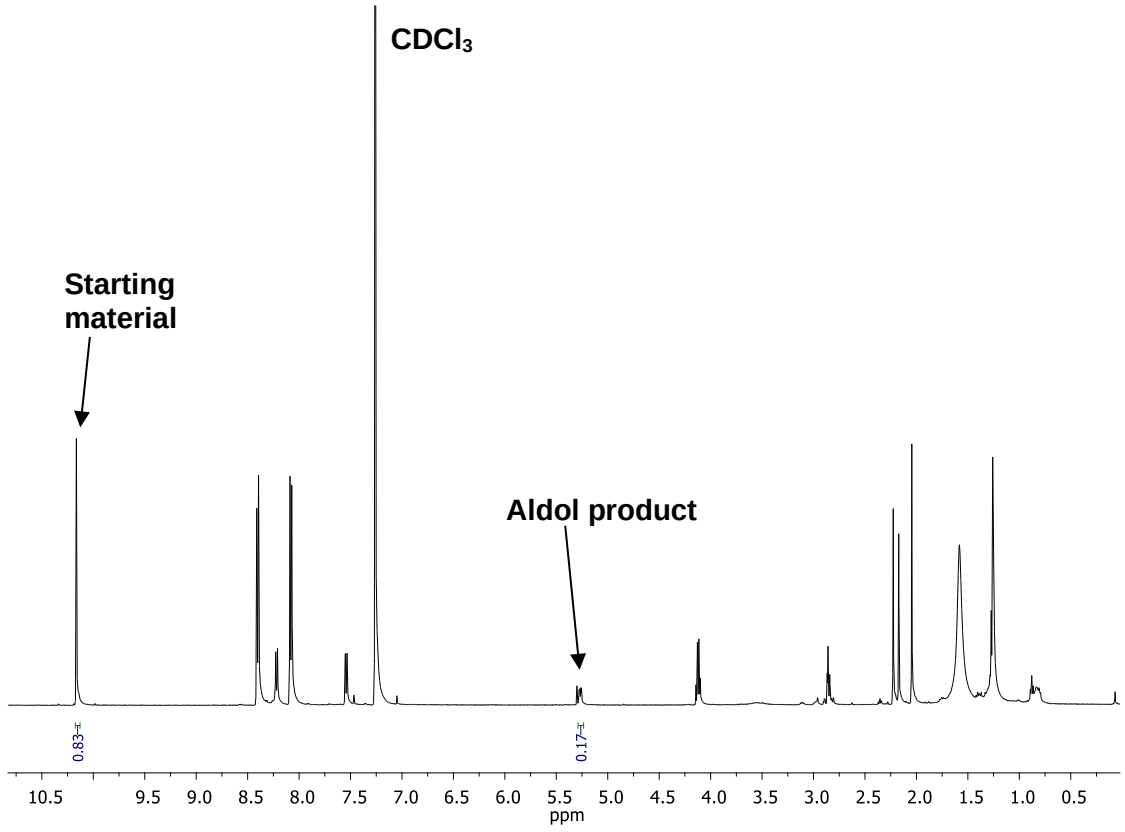


Entry	Retention time (min)	Peak Area (%)
1 (*R*-enantiomer)	16.2	56.2
2 (*S*-enantiomer)	17.6	43.8

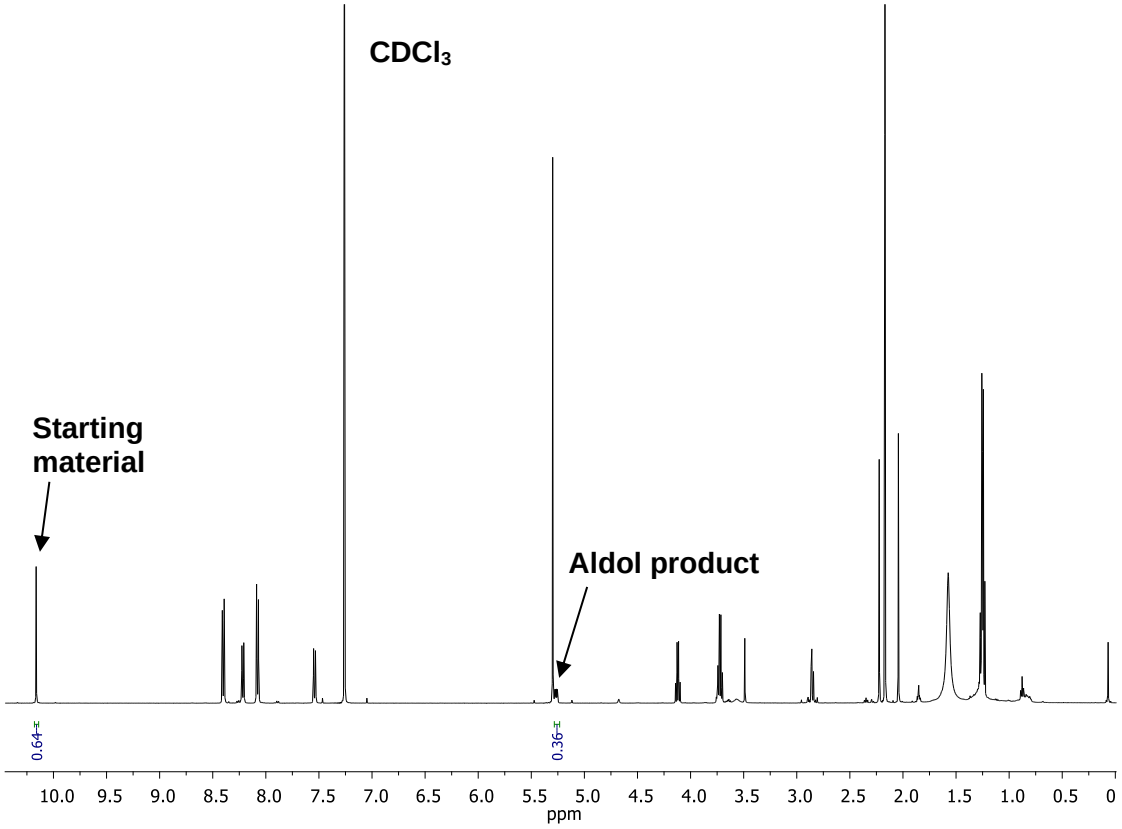
6 ^{1}H NMR Details for the Activity Screening of Catalysts 1 for the Aldol Reaction of Acetone and *p*-Nitro Benzaldehyde

6.1 Screening Reactions

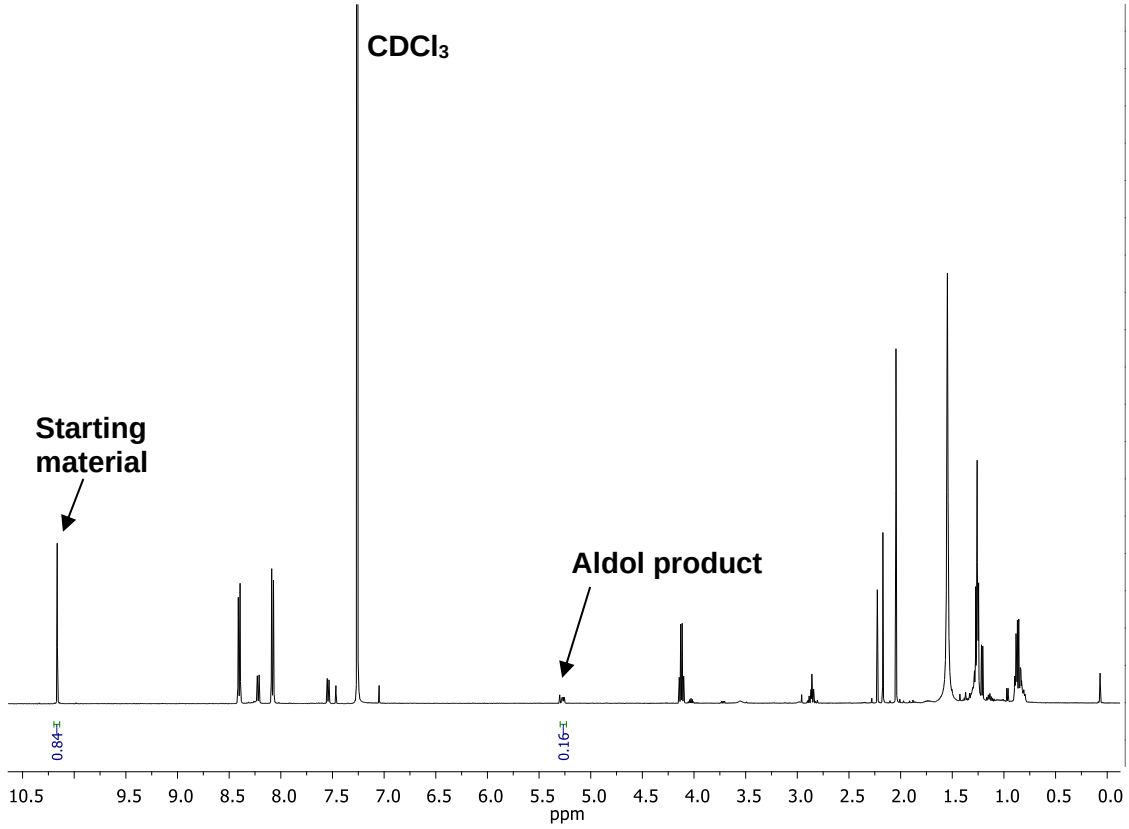
6.1.1 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and Acetone (no catalyst)



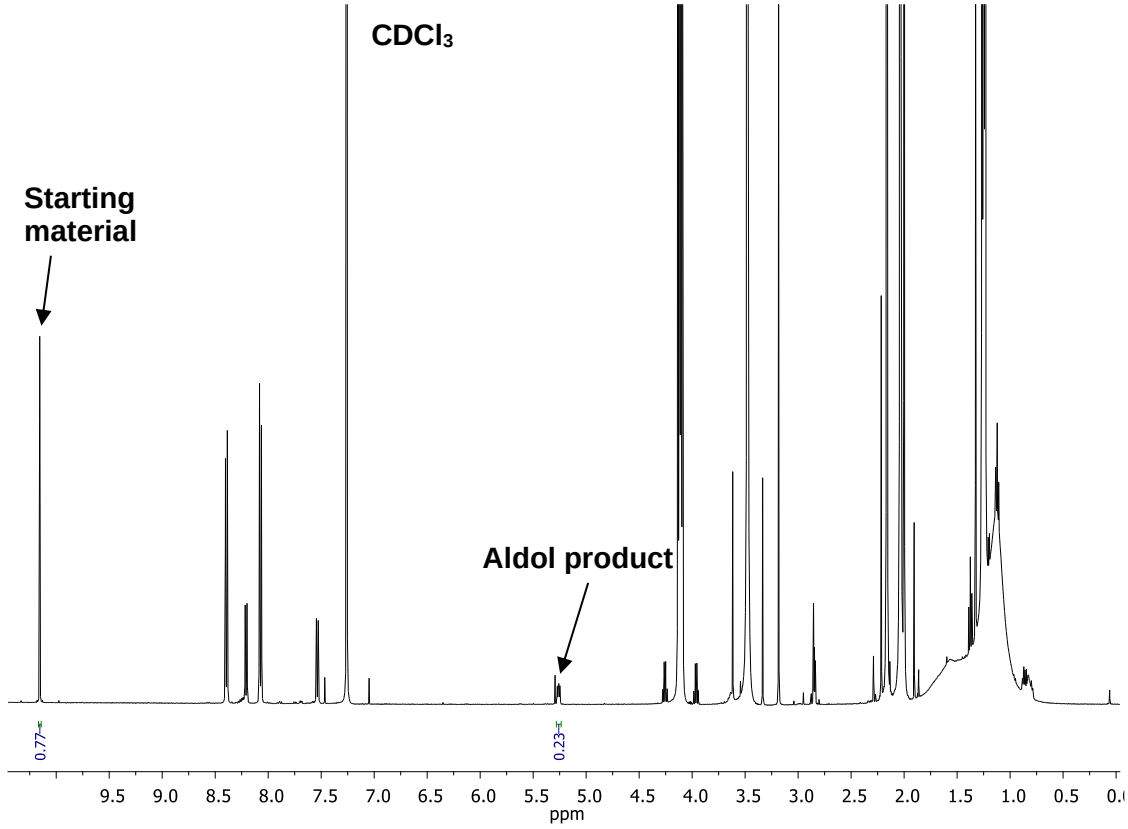
6.1.2 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and Acetone (catalyst 1)



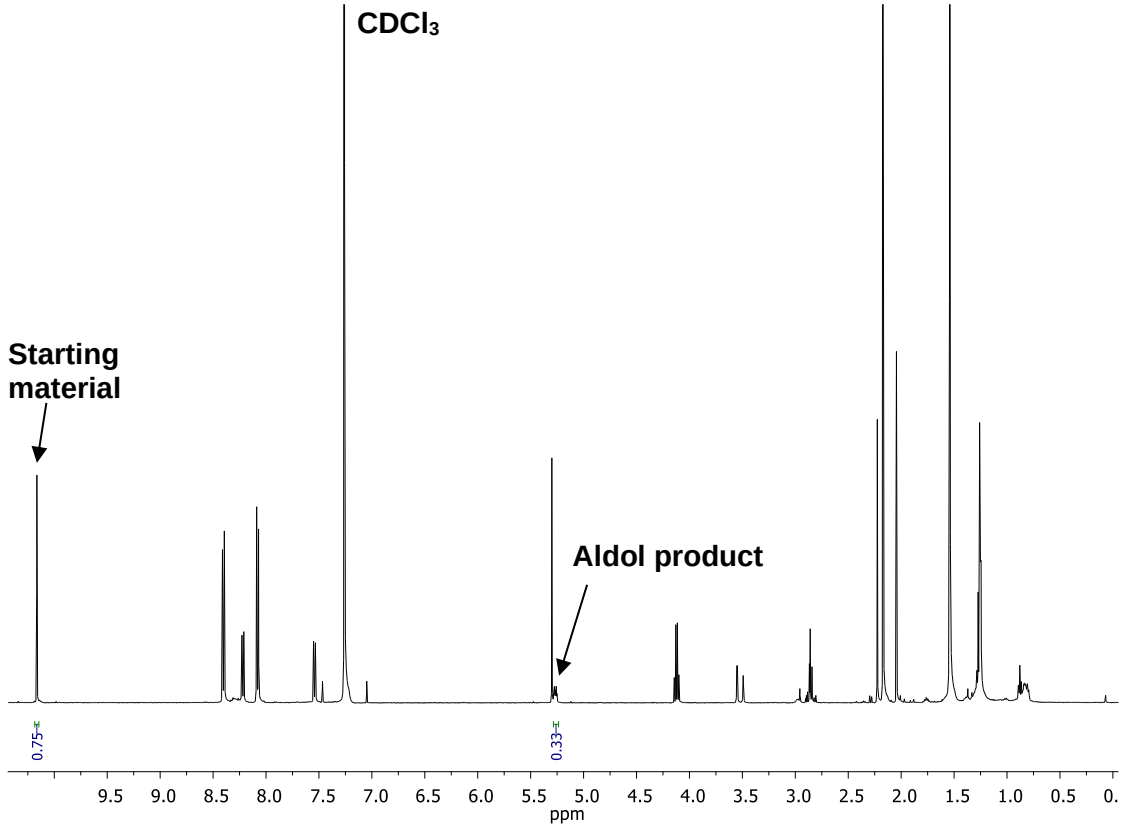
6.1.3 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and Acetone (Sav, 0.1 mol%)



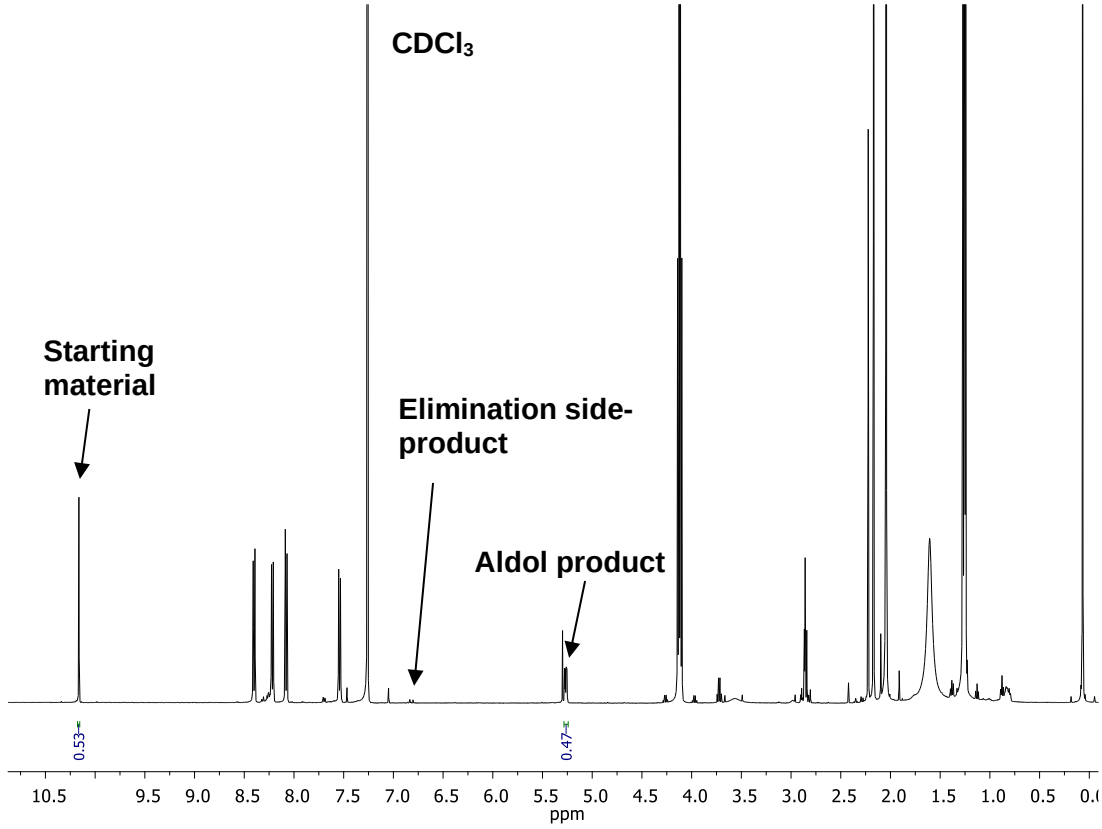
6.1.4 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and Acetone (Sav, 0.5 mol%)



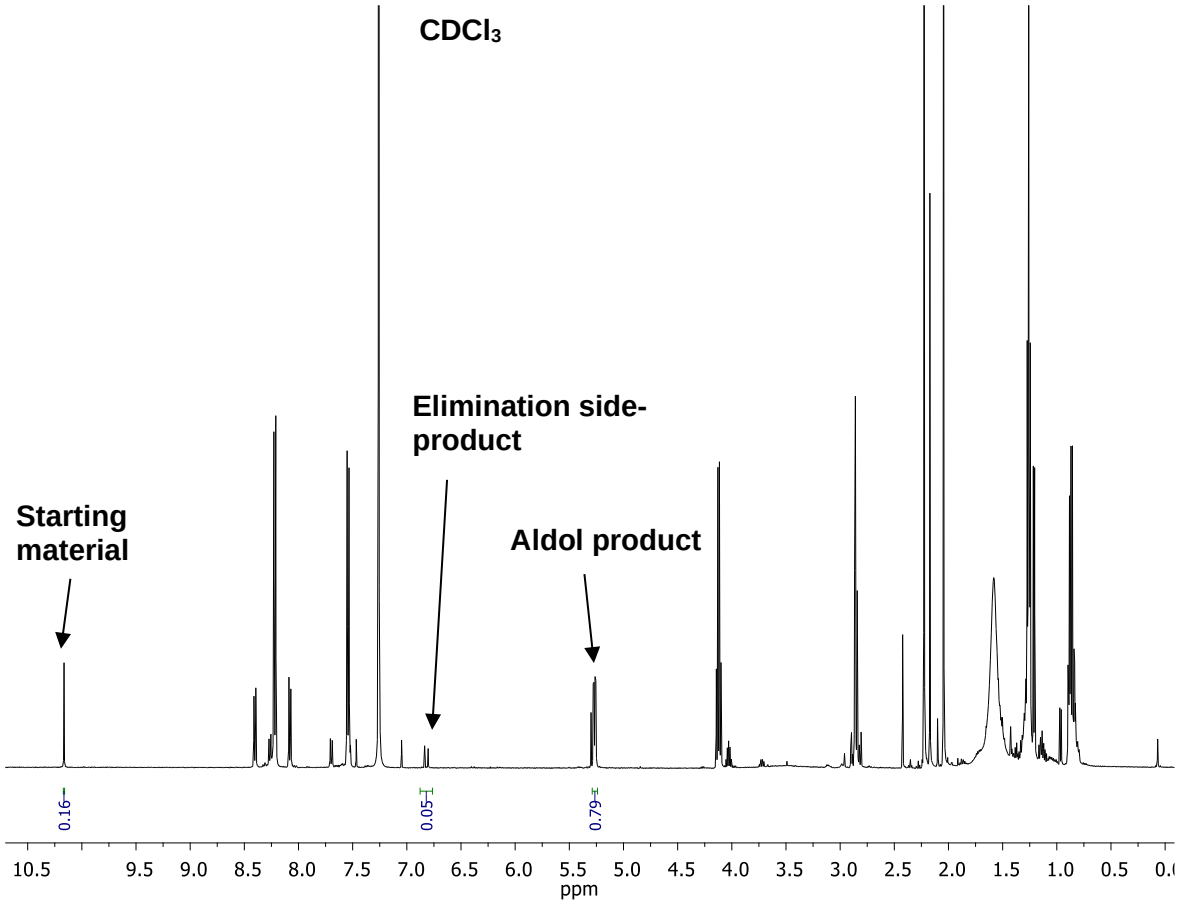
6.1.5 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and Acetone (Sav, 1 mol%)



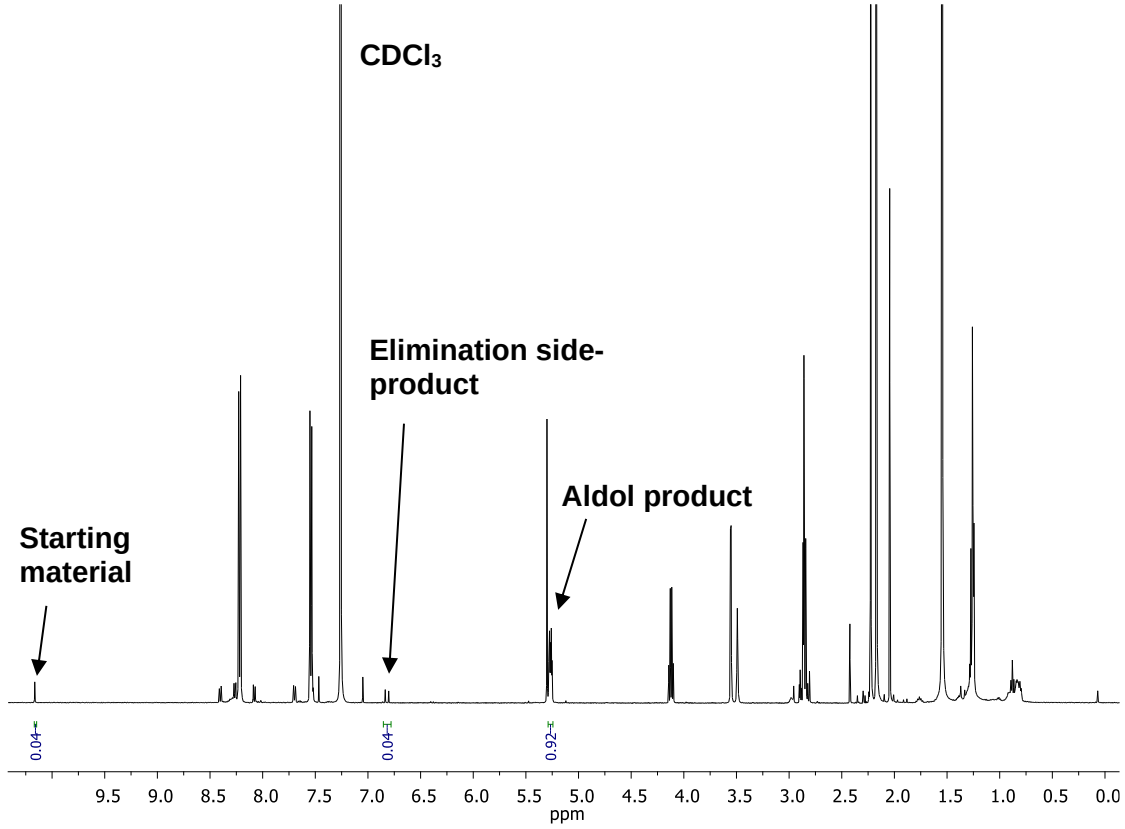
6.1.6 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and Acetone (Sav:1, 0.1 mol%)



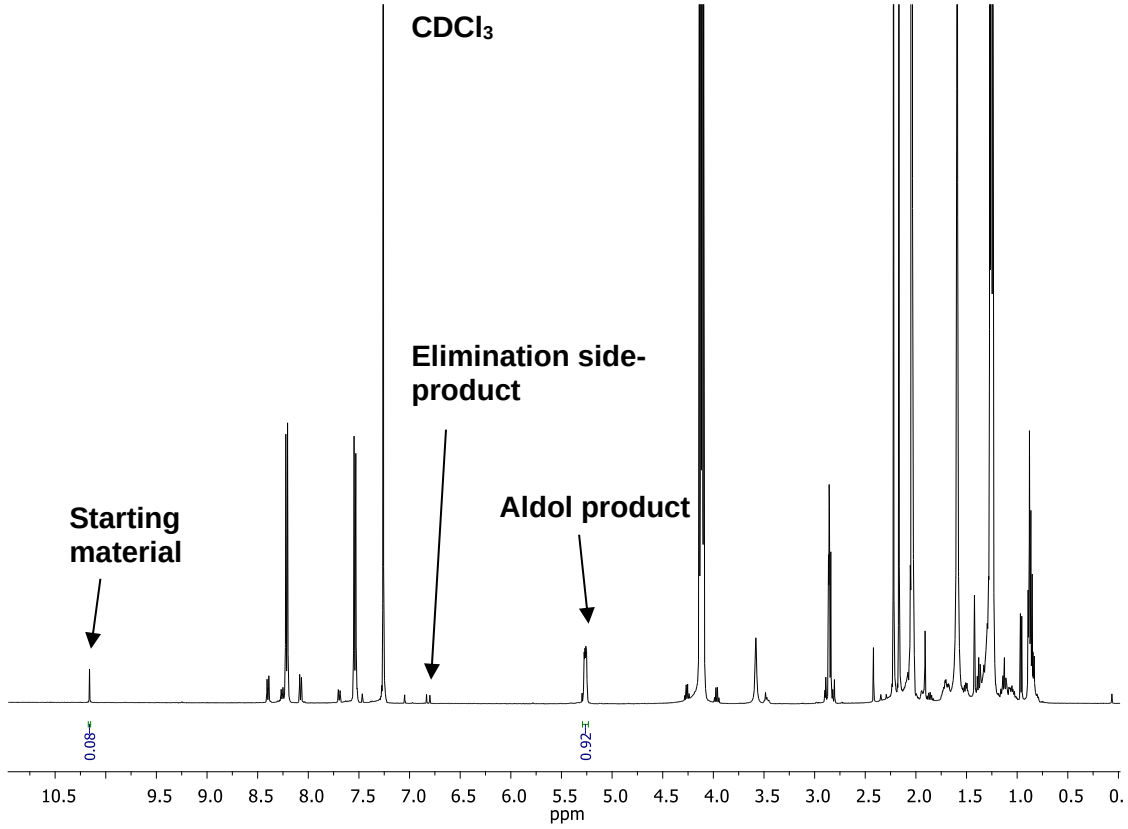
6.1.7 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and Acetone (Sav:1, 0.5 mol%)



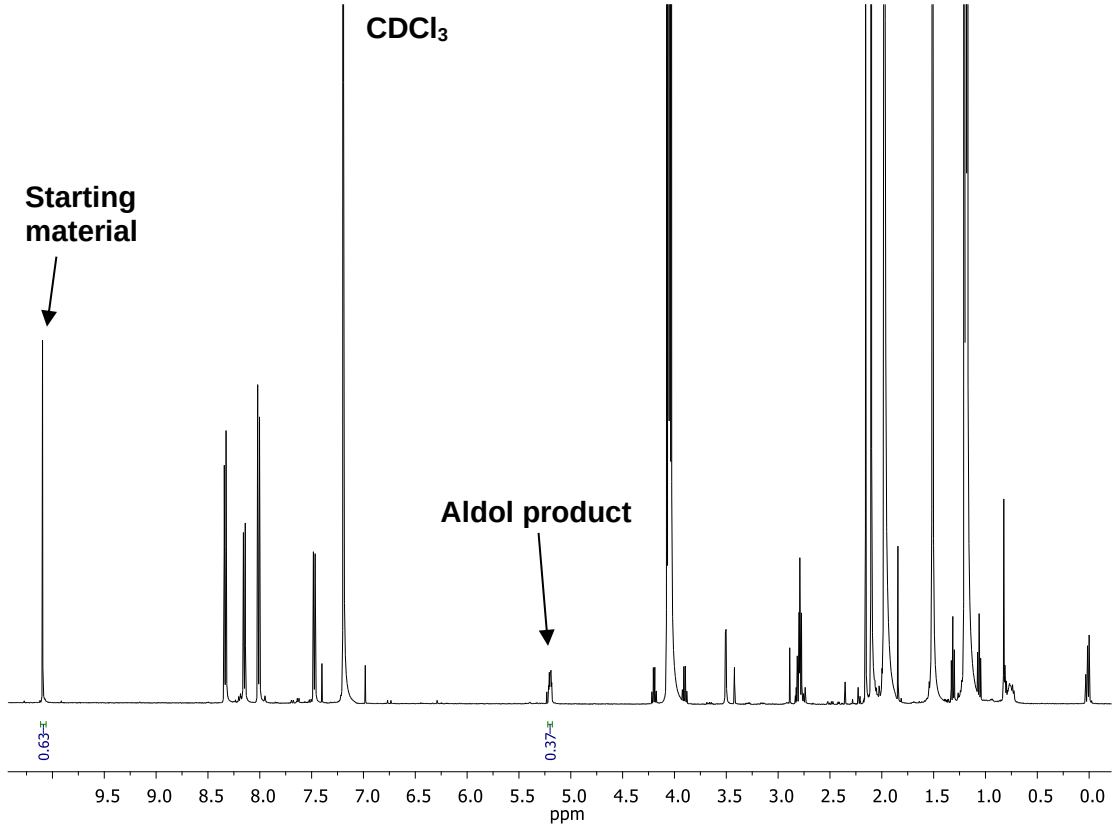
6.1.8 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and Acetone (Sav:1, 1 mol%)



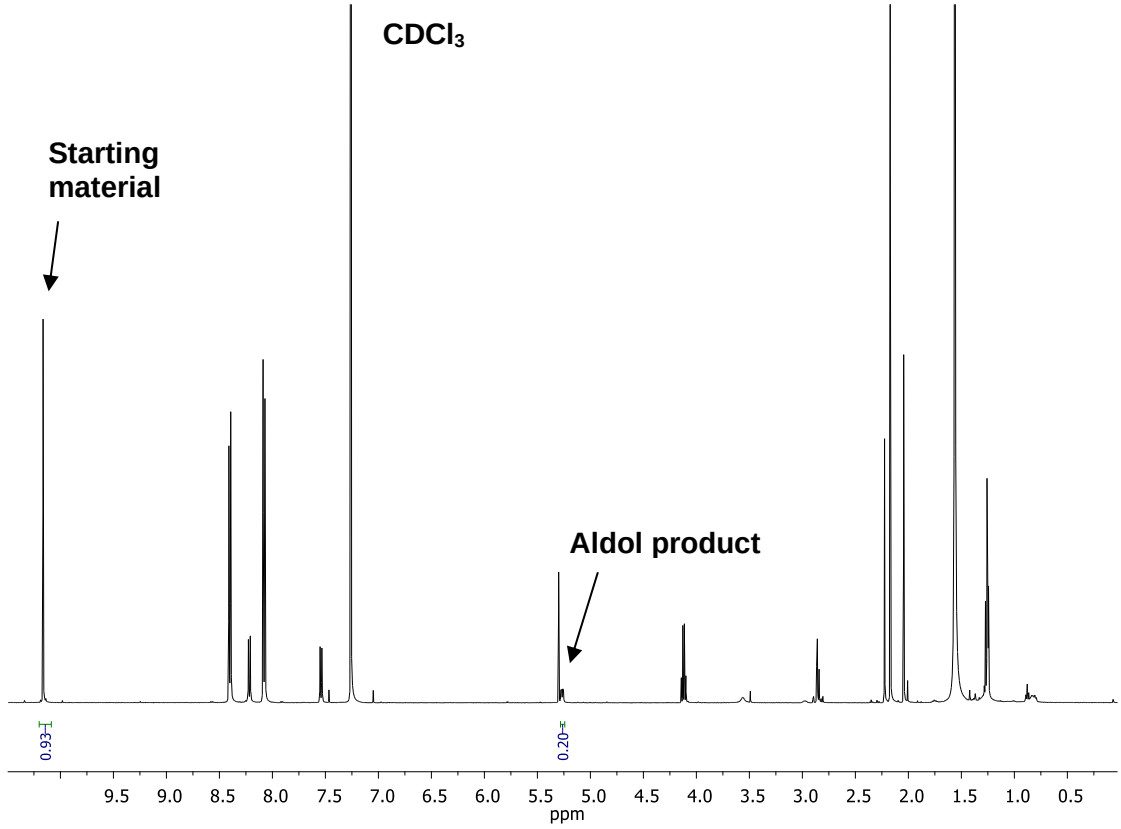
6.1.9 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and Acetone (Sav:1, 1 mol%, and TFA 1 mol%)



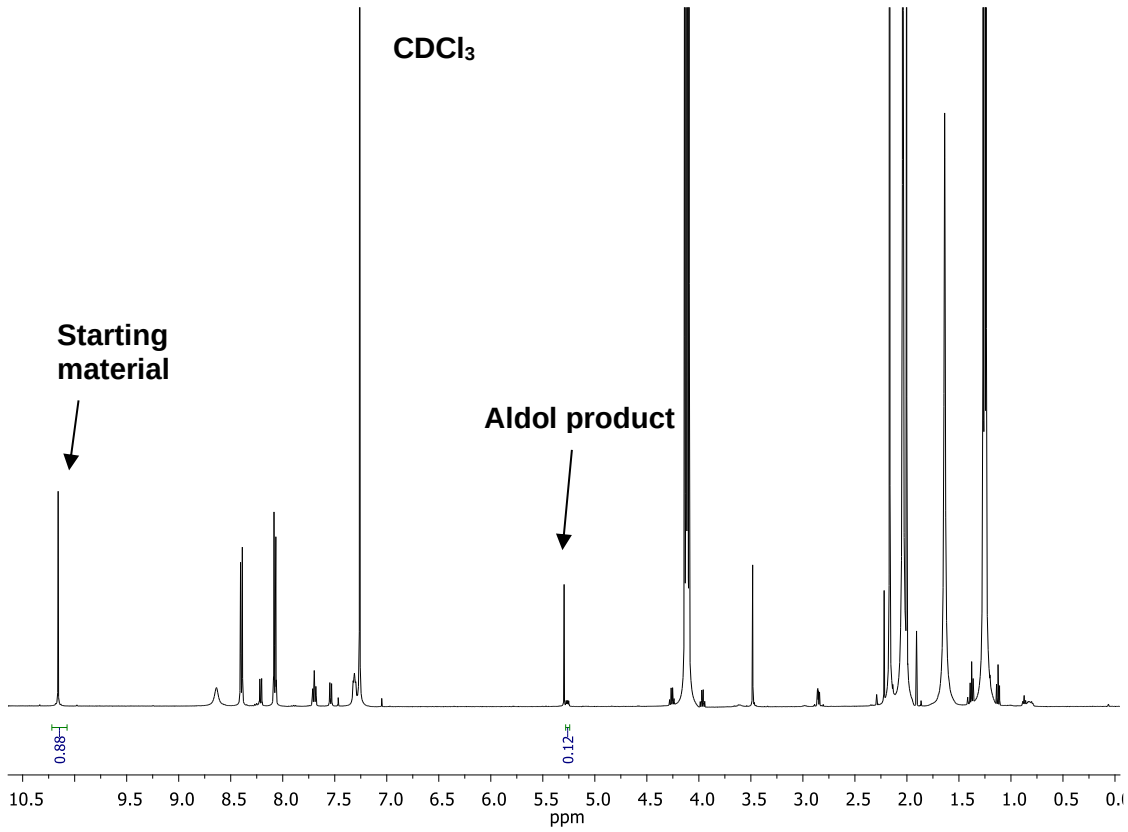
6.1.10 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and Acetone (Sav:1, 1 mol%) at 10 °C



6.1.11 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and 5 Equivalent of Acetone (Sav:1, 0.1 mol%, 25% Methanol)

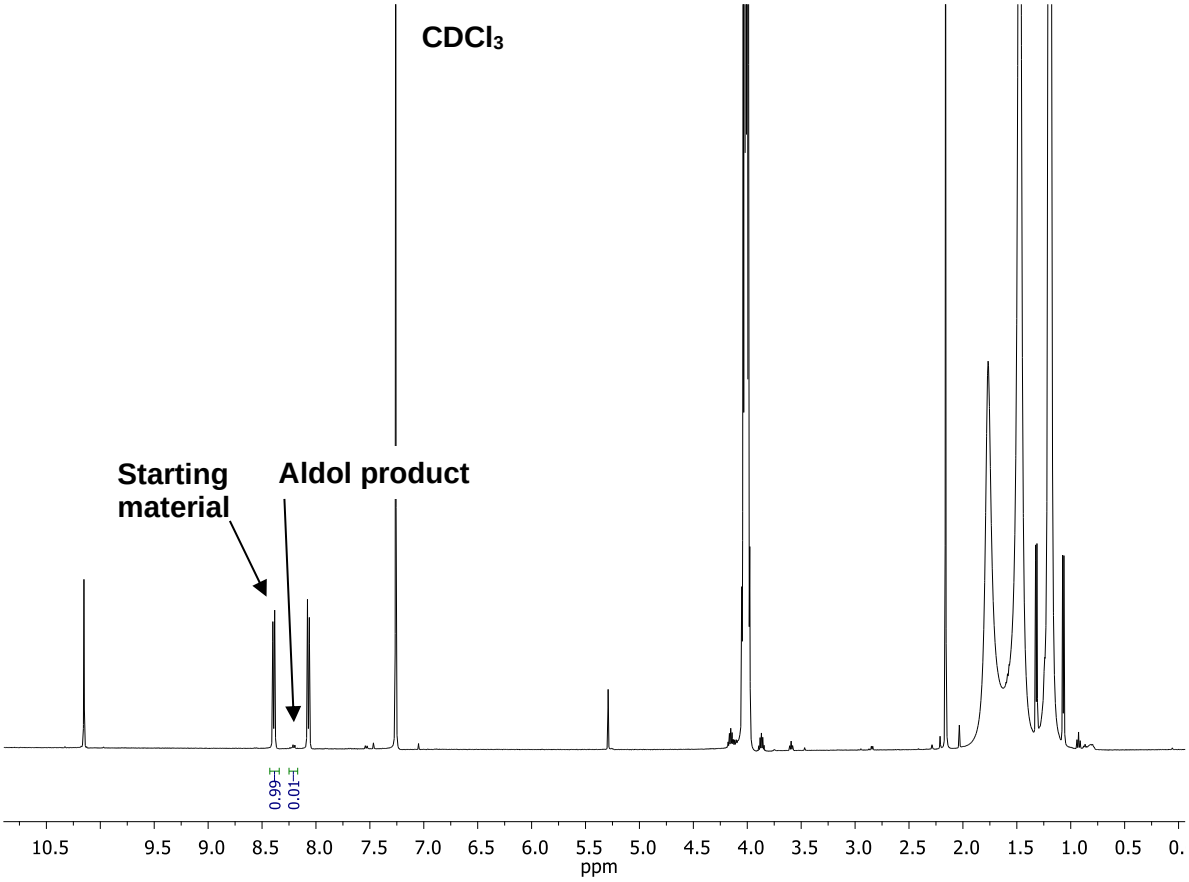


6.1.12 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and 5 Equivalent of Acetone (Sav:1, 0.1 mol%, 25% Acetonitrile)



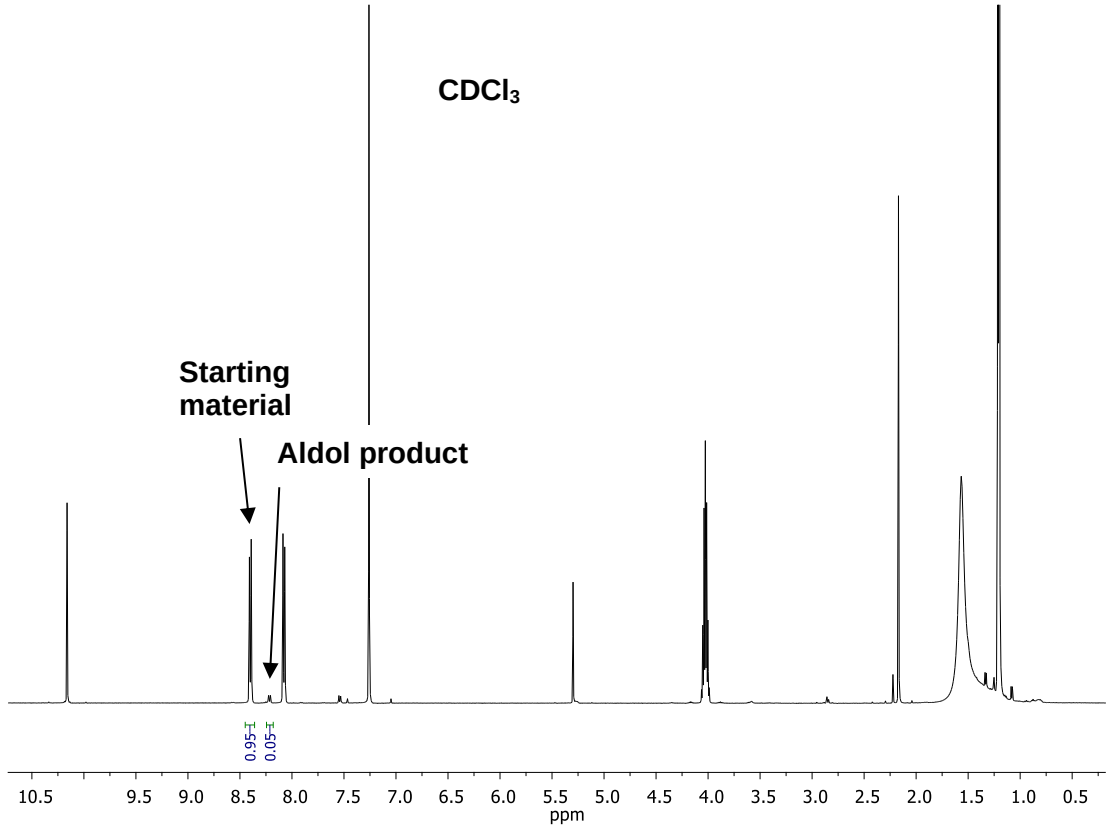
6.1.13 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and 5 Equivalent of Acetone (Sav:1, 0.1 mol%, 25% Iso-Propanol)

Integrations of starting material and product has been made using the aromatic peaks as standard.

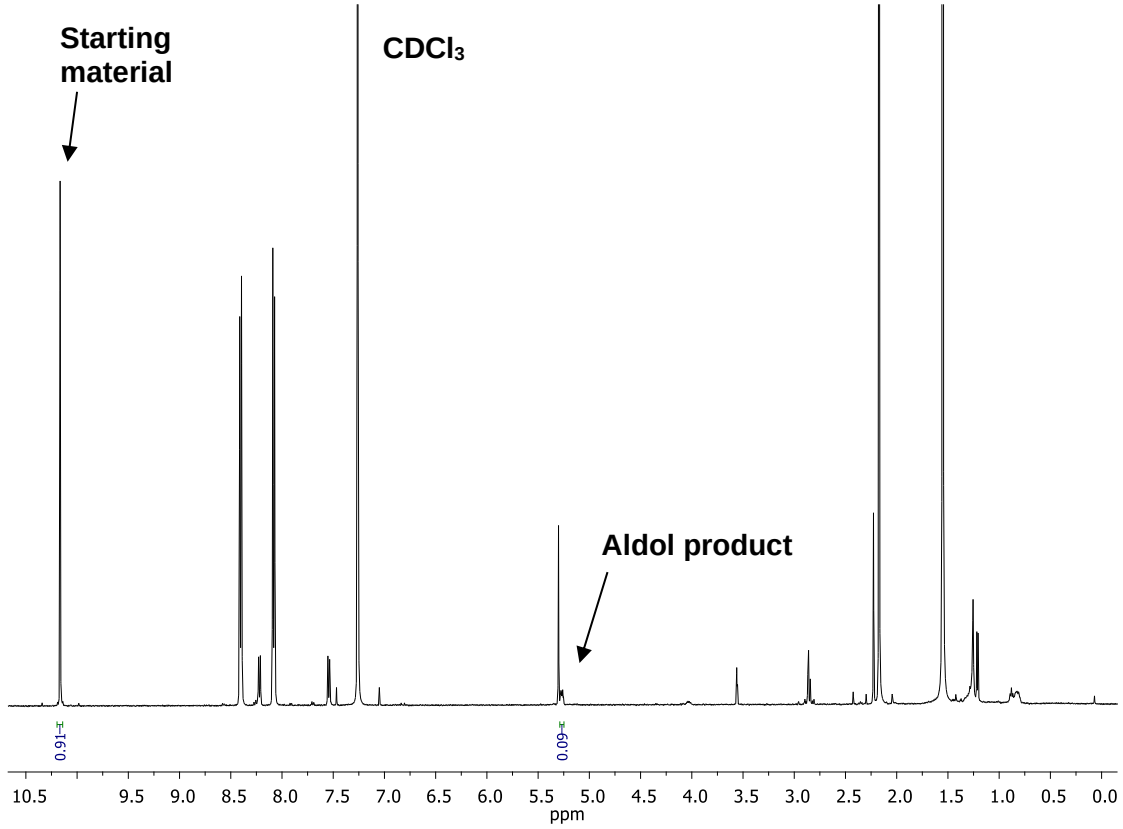


6.1.14 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and 10 Equivalent of Acetone (Sav:1, 0.1 mol%, 25% Iso-Propanol)

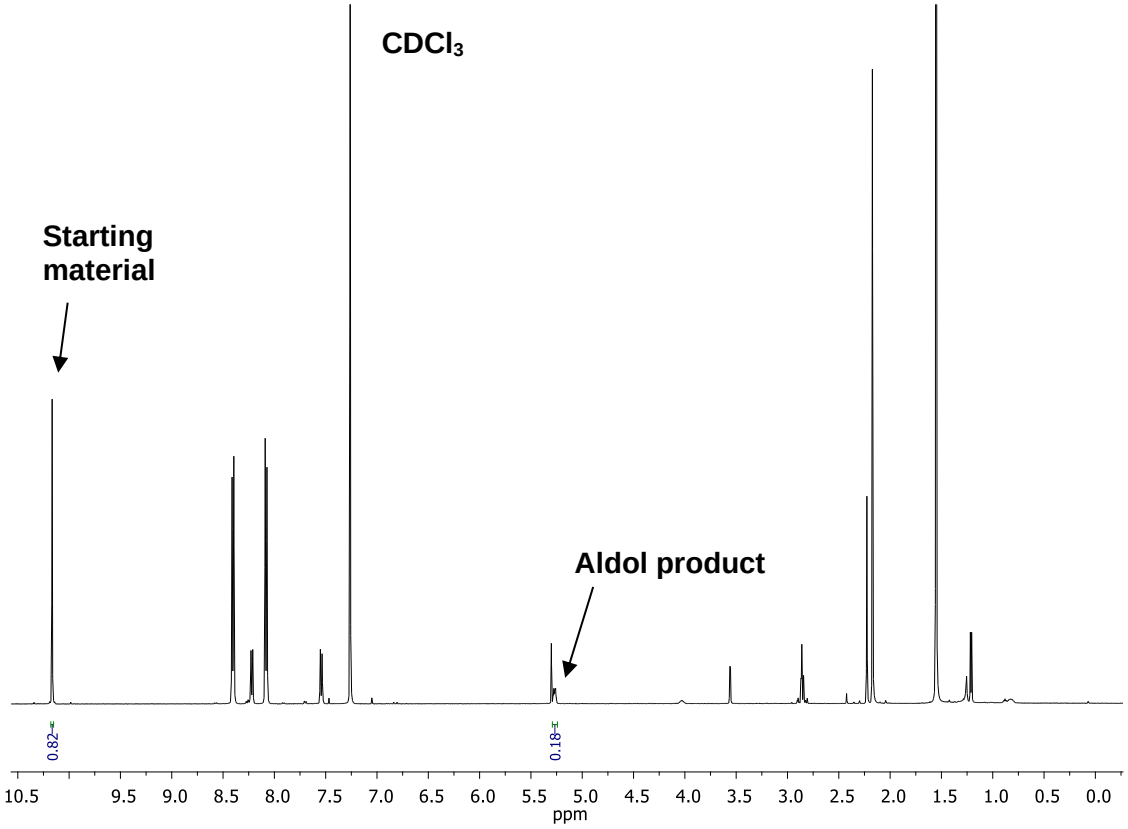
Integrations of starting material and product has been made using the aromatic peaks as standard.



6.1.15 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and 25 Equivalent of Acetone (Sav:1, 0.1 mol%, 25% Iso-Propanol)

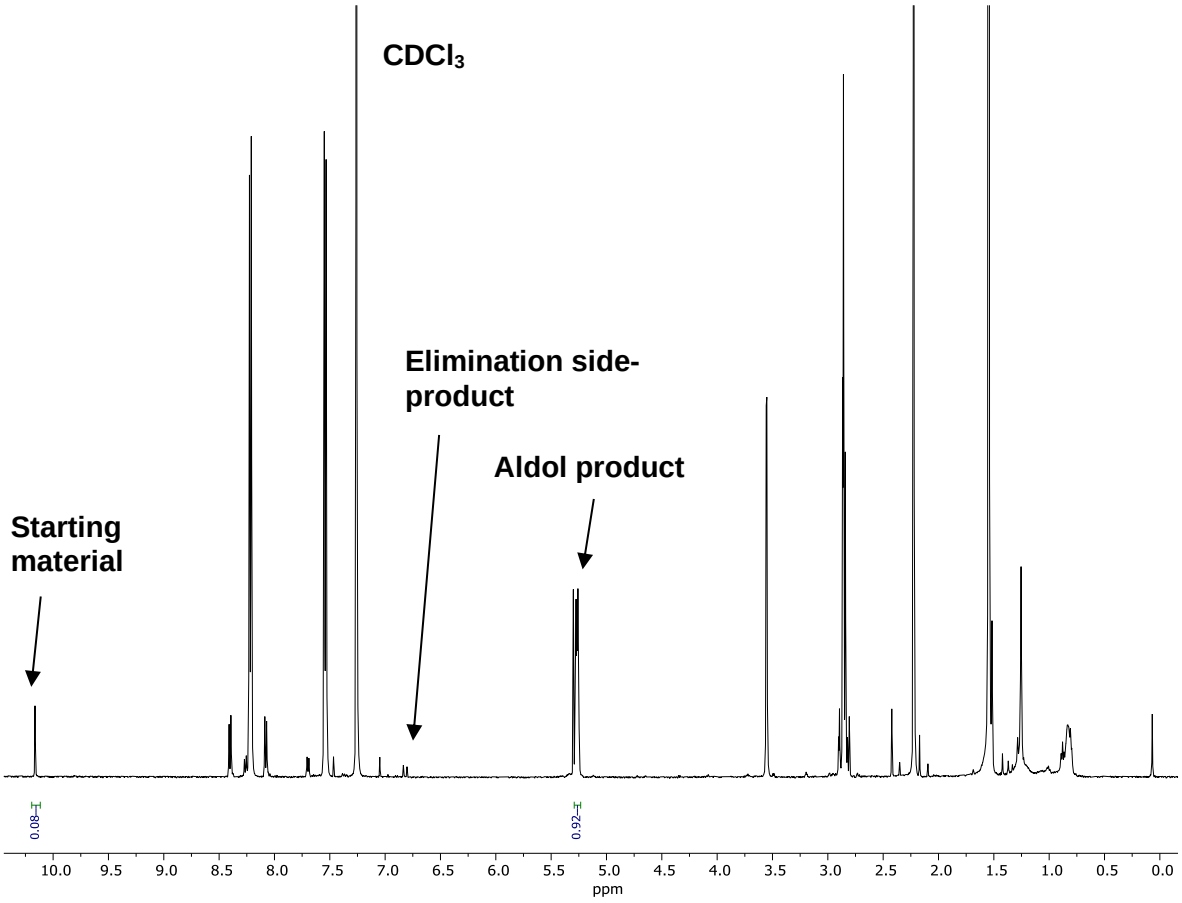


6.1.16 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and 50 Equivalent of Acetone (Sav:1, 0.1 mol%, 25% Iso-Propanol)

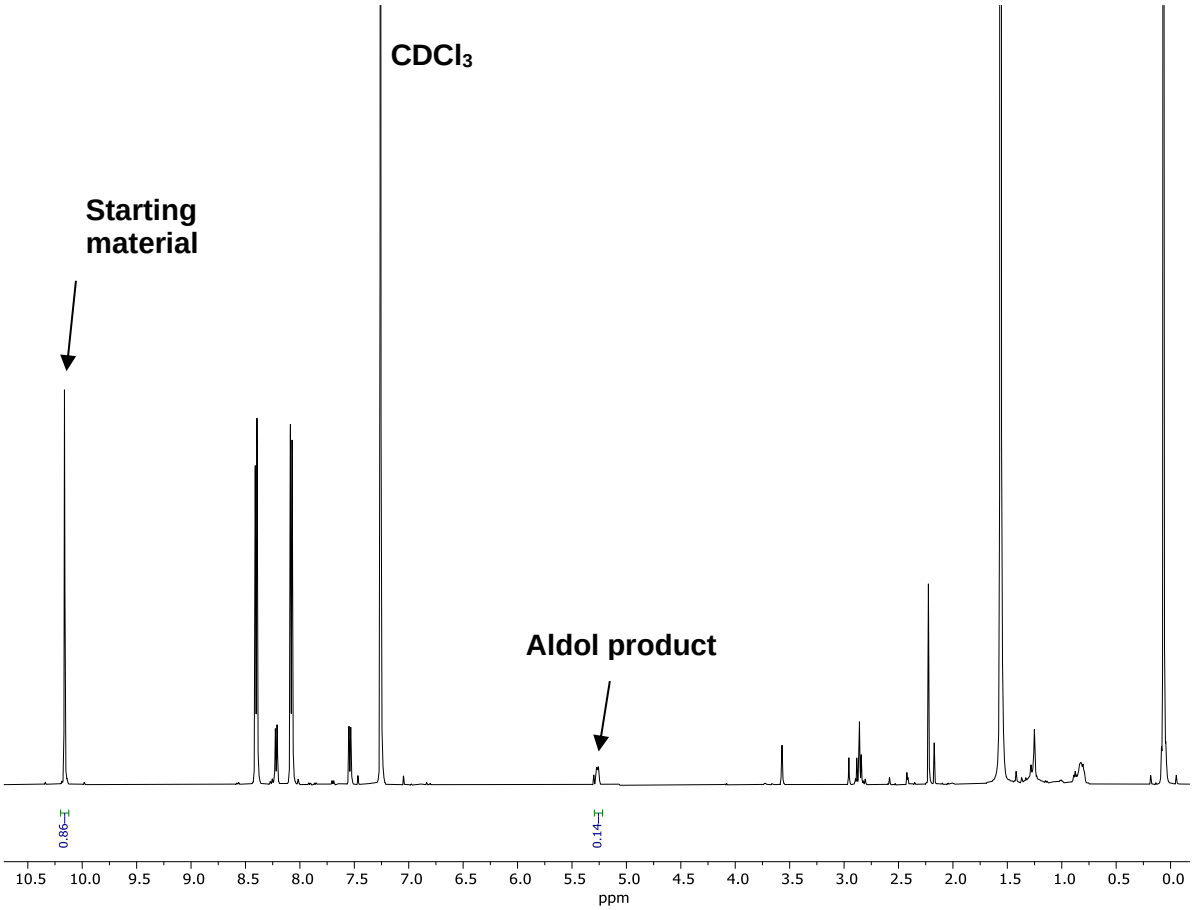


6.2 Protein scope

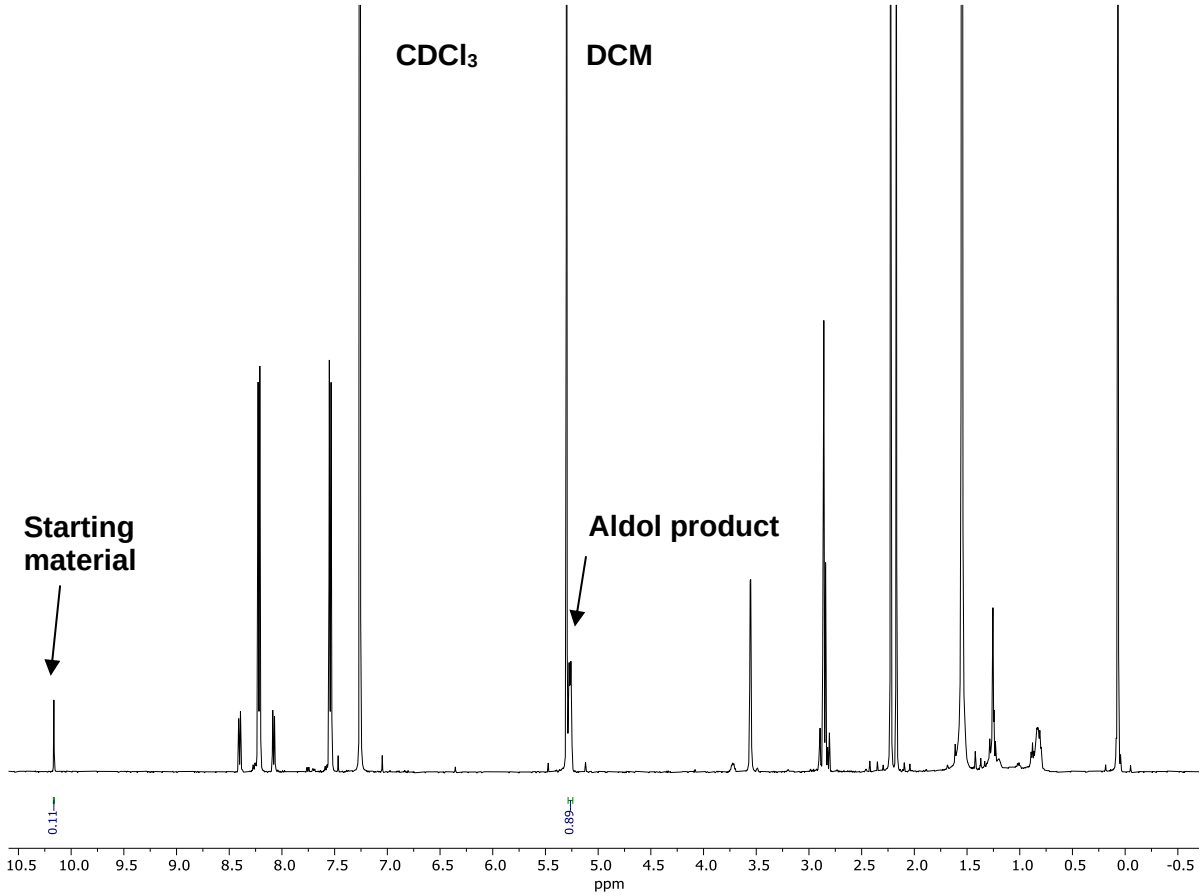
6.2.1 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and Acetone (T-rSav:1, 1 mol%)



6.2.2 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and Acetone (S112E:1, 1 mol%)



6.2.3 ^{1}H NMR Spectrum after Extraction of the Reaction Mixture between *p*-Nitro Benzaldehyde and Acetone (K121A:1, 1 mol%)



7 References

[1] T. Sano, W. M. Pandori, X. Chen, L. C. Smith, R. C. Cantor, *J. Biol. Chem.* **1995**, *270*, 28204-28209.
[2] M. Hosseini, N. Stiasni, V. Barbieri, C. O. Kappe, *J. Org. Chem.* **2007**, *72*, 1417-1424.