Electronic Supplementary Material (ESI) for Green Chemistry.

A biocatalytic cascade for the conversion of fatty acids to fatty amines

Supporting information

Joan Citoler,[a] Sasha R. Derrington,[a] James L. Galman,[a] Han Bevinakatti,[b,*] Nicholas J. Turner[a,*]

[a]*Manchester Institute of Biotechnology, The University of Manchester,131 Princess Street, Manchester, M1 7DN, United Kingdom. E-mail: nicholas.turner@manchester.ac.uk*
[b] *AkzoNobel Surface Chemistry, 10 Finderne Ave, Bridgewater, NJ 08807, USA*

Contents

General information

Materials

Commercially available reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK), Alfa Aesar (Karlsruhe, Germany), Thermofischer (Waltham, MA, USA), Prozomix (Haltwhistle, Northumberland, UK), New England Biolabs (Ipswich, MA, USA) and Codexis (Redwood City, CA, USA). They were used in commercial conditions without any alteration.

Biocatalysts information and preparation

Molecular biology

Mycolicibacterium chlorophenolicum carboxylic acid reductase (*Mc*CAR) gene was cloned from genomic DNA (**Table S1**). The rest of genes were ordered from GeneArt Strings™ (Thermofischer, USA) following codon optimization. In-Fusion® HD cloning kit from Takara Bio USA, Inc. (CA, USA) was used to clone the fragments into pET expression vectors. *In silico* experimental planning using the SnapGene software facilitated primer design. pET vectors were linearised either by PCR or using restriction enzymes and genes were amplified by PCR to incorporate overlapping regions (In the case of *Mc*CAR, amplification was performed by colony PCR). Cloning success was confirmed by sequencing the whole gene using T7 promoter and terminator primers.

Primer name	Oligo sequence (5'-3')
***Mc*CAR Forward**	ACGCAGCATATGCCGACTGAAACCCGCGAC
***Mc*CAR Reverse**	GTGGCTGAATTCTCAGAGCAAGCCAAGCAGTTCG

Table S1 Primers used for amplification of McCAR gene in *Mycolicibacterium chlorophenolicum*

Expression

E. coli BL21 (DE3) chemically competent cells were transformed with plasmids containing the gene sequences encoding the enzymes of interest (**Table S2**). Transformed cells were grown overnight at 37 °C and 200 rpm in 50mL falcon tubes containing 20mL of LB medium (1% tryptone, 0.5% yeast extract, 1%NaCl) with appropriate antibiotics. The day after, 2L baffled flasks supplemented with 600mL LB medium with appropriate antibiotic were inoculated with 1:100 dilution of overnight culture. At OD 0.6-0.8, cells were inoculated with IPTG to a final concertation of 1 mM and incubated overnight at 20 °C and 200 rpm. Cells were harvested by centrifugation at 4000 rpm for 20 min and stored at -20 °C for further use.

Enzyme	Organism	Accession no.	Plasmid
*Mc*CAR	*Mycolicibacterium chlorophenolicum*	WP_048469799.1	pET28b
*Sr*CAR	*Segniliparus rugosus*	WP_007468889	pET28b
*Mm*CAR	*Mycobacterium marinum*	WP_012393886	pET21b
*Ni*CAR	*Nocardia iowensis*	AAR91681	pET21b
Cv-TA	*Chromobacterium violaceum*	WP_011135573.1	pET28b
Pp-SpuC	*Pseudomonas putida NBRC 14161*	9494	pET22b
Vf-TA	*Vibrio fluvialis*	AEA39183.1	pET28b
ATA-117	*Arthrobacter sp.*	3WWH_A	pET21b
At-TA	*Agrobacterium tumefaciens*	WP_010972924.1	pET28b
Sp-TA	*Silicibacter pomeroyi*	WP_011049154.1	pET28b
Sfp	*Bacilus subtilis*	WP_101501862.1	pCDF-1b
PAP	*Acinetobacter johnsonii*	WP_114836924.1	pET28b
AdK	*Acinetobacter johnsonii*	WP_058869236.1	pET28b

Table S2 Biocatalysts employed in biocatalytic tandem cascade reaction.

Lysate preparation

Cell pellets were resuspended in the desired reaction buffer at a concentration of 200 mg_{cww}/mL. Subsequently, cells were lysed by ultra-sonication (20 s ON, 20s OFF, 20 cycles) using a Soniprep 150 (MSE UK Ltd.) and centrifuged at 18,000 rpm for 30 min to remove the cell debris. Supernatant was snap-frozen in liquid N_2 and stored at -80 °C for further use.

Pure enzyme preparation

Cell pellets were resuspended in 20mL of Buffer A (100 mM Kpi, 300mM NaCl, pH 7.8) and lysed by ultra-sonication (20 s ON, 20s OFF, 20 cycles) using a Soniprep 150 (MSE UK Ltd.). Subsequently, cell lysates were subjected to centrifugation at 18,000 rpm for 30min to remove cell debris. Supernatant was filtered through a 0.45 um microfilter (Sartorius GmbH) and loaded onto a 5 mL His-Trap HP column (GE Healthcare) previously equilibrated with 10 column volumes of buffer A in the AKTA purification system (GE Healthcare). Column was washed with 10 column volumes of 2.5% and 5% Buffer B (100mM Kpi, 300mM NaCl, 1M Imidazole) in buffer A. Protein was eluted with 15 column volumes of an increasing gradient of buffer B in buffer A (5-100%). Collected fractions containing the pure protein were loaded into Sephadex G-25 PD-10 Desalting columns and buffer was exchanged to our desired buffer for the reaction. Buffer exchanged fractions were snap-frozen with liquid N_2 and stored at -80 °C for further use. SDS-page analisys of the pure fractions as well as lysate can be seen in table 1.

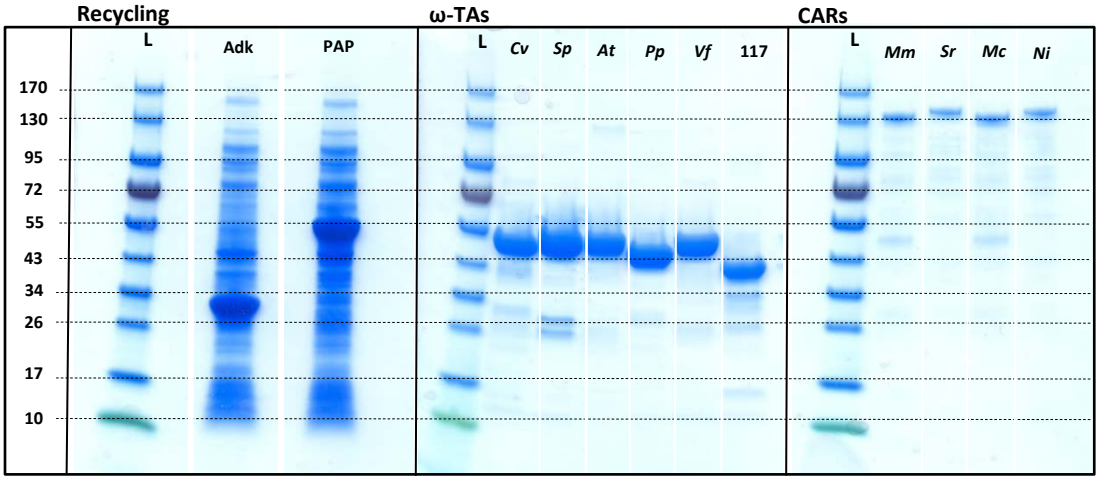


Figure S1 SDS-PAGE for the expression and purification of the enzymes used in the primary crossed combination between CARs and TAs, together with PAP and Adk cell free extracts.

Analytical methods

GC-FID Analysis

GC analysis was conducted with an Agilent 6850 GC (Agilent, Santa Clara, CA, USA) with a flame ionization detector (FID) using a 30 m long HP-1 column with 0.320 mm of diameter and a film of 0.25 µm (Agilent, Santa Clara, CA, USA). Two methods were used for identification and quantification of all the compounds present in the biotransformations, which can be found in table 3;

Method 1 (low boiling point):
Inlet temperature: 250 ºC
Detector temperature: 250 ºC
Column conditions: 7.1 psi, He flow 1.3 mL/min. Starting T: 60 ºC held for 3 min. 20 ºC/min rate to 220 ºC. 40 ºC/min rate to 300 ºC.

Method 2 (high boiling point):
Inlet temperature: 300 ºC
Detector temperature: 300 ºC
Column conditions: 9.9 psi, He flow 1.3 mL/min. Starting T: 150 ºC held for 1 min. 10 ºC/min rate to 200 ºC. 30 ºC/min rate to 320 ºC. 320 ºC held for 5 min.

Compound	Method	Retention time (min)
1-hexylamine	Method 1	4.142
1-hexanol	Method 1	4.432
Hexanoic acid	Method 1	6.315
1-octylamine	Method 1	6.440
1-octanol	Method 1	6.660
Octanoic acid	Method 1	7.800
1-decylamine	Method 2	2.570
1-decanol	Method 2	2.640
Decanoic acid	Method 2	3.300
1-dodecylamine	Method 2	3.700
1-dodecanol	Method 2	3.810
Dodecanoic acid	Method 2	4.430
1-tetradecylamine	Method 2	5.304
1-tetradecanol	Method 2	5.448
Myristic acid	Method 2	6.024
1-hexadecylamine	Method 2	7.200
1-hexadecanol	Method 2	n.d.
Palmitic acid	Method 2	8.076
1-octadecylamine	Method 2	8.777
1-octadecanol	Method 2	n.d.
Stearic acid	Method 2	9.258
Oleyl amine	Method 2	8.611
Oleyl alcohol	Method 2	n.d.
Oleic acid	Method 2	9.138
Linoleyl amine	Method 2	8.560
Linoleyl alcohol	Method 2	n.d.
Linoleic acid	Method 2	9.094
Linolenyl amine	Method 2	8.587
Linolenyl alcohol	Method 2	n.d.
Linolenic acid	Method 2	9.096

Table S3 Retention times and method used for each of the compounds identified and quantified.

GC-MS Analysis

GC-MS analysis for identification of the fatty amines which were not in our possession was conducted with an Agilent 7890B (Agilent, Santa Clara, CA, USA) containing an Agilent 5977B Mass Selective Detector and an Agilent 7693 autosampling robot. The system was equipped with a 30m long HP-1MS column with 0.320 mm of diameter and a film of 0.25 µm (Agilent, Santa Clara, CA, USA). The method used for identification was the following:

GC-MS method:
Inlet temperature: 270 °C
Detector temperature: 270 °C
Column conditions: 4.6 psi, He flow 2 mL/min. Starting T: 50 °C held for 2 min. 7 °C/min rate to 70 °C. 30 °C/min rate to 320 °C, held for 2 min.

Biotransformation

Analytical scale biotrasformations were conducted in 2mL HPLC vials containing 500 µL reaction mixture (aqueous phase) and 500 µL of *n*-heptane (organic phase). Reactions were performed at 30 °C, 190 rpm for 20 h and extracted 3 times by adding 40 µL 10M NaOH to reach pH 12 followed by 750 µL of n-heptane. After vigorous shaking and centrifugation at 4000 rpm during 5 min, 1 mL of organic phase was extracted for each of the times. The organic extracts were combined together dried over $MgSO_4$ before analysis by GC-FID. Product conversion was calculated through a calibration curve. For the substrate scope screening, reactions were first acidified with 40 µL 5M HCl to extract the acid and subsequently basified with 80 µL 10M NaOH to extract the amine. Organic extracts containing acid and amine were combined and conversion was calculated through GC-FID traces.

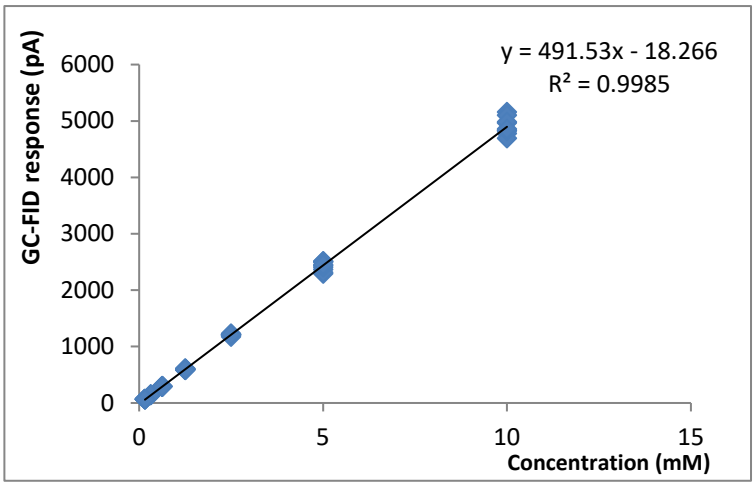


Figure S2. Calibration curve for dodecylamine.

Combination of CARs and TAs

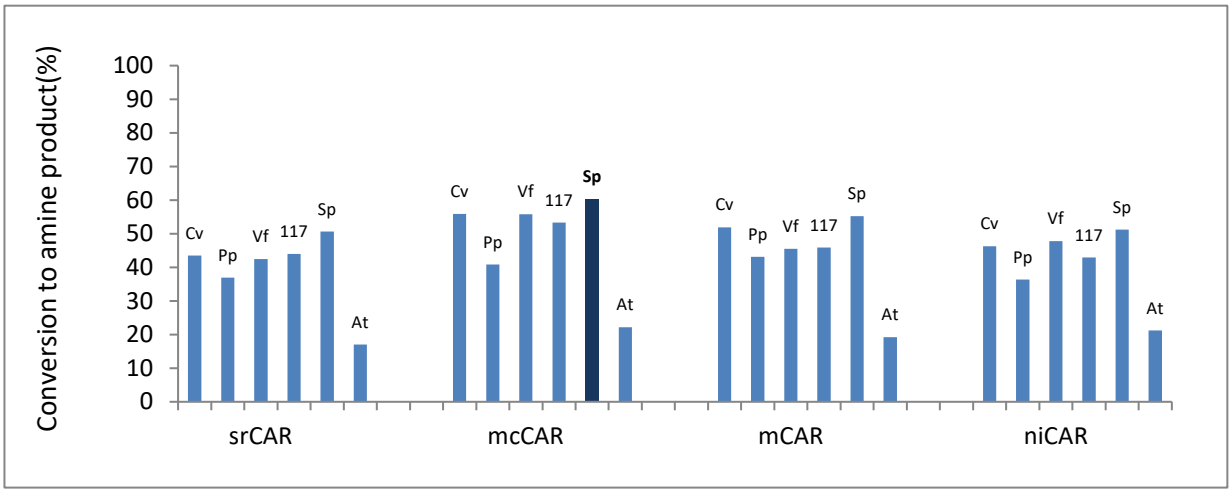


Figure S3. Combinatorial screen between CARs and ω-TAs. Reaction conditions: 5 mM dodecanoic acid, 50 mM $MgCl_2$, 50 mM D-glucose, 0.5 mM NADP+, 250 mM IPA, 1 mg mL^{-1} CAR, 1 mg mL^{-1} ω-TA, 2 mg mL^{-1} PAP lysate, 2 mg mL^{-1} Adk lysate, 0.2 mg mL^{-1} CDX-901 GDH, 5% (v/v) DMSO, 100 mM Tris-HCl pH 8, 500µL n-heptane (1:1 v/v to aqueous phase).

Reaction intensification

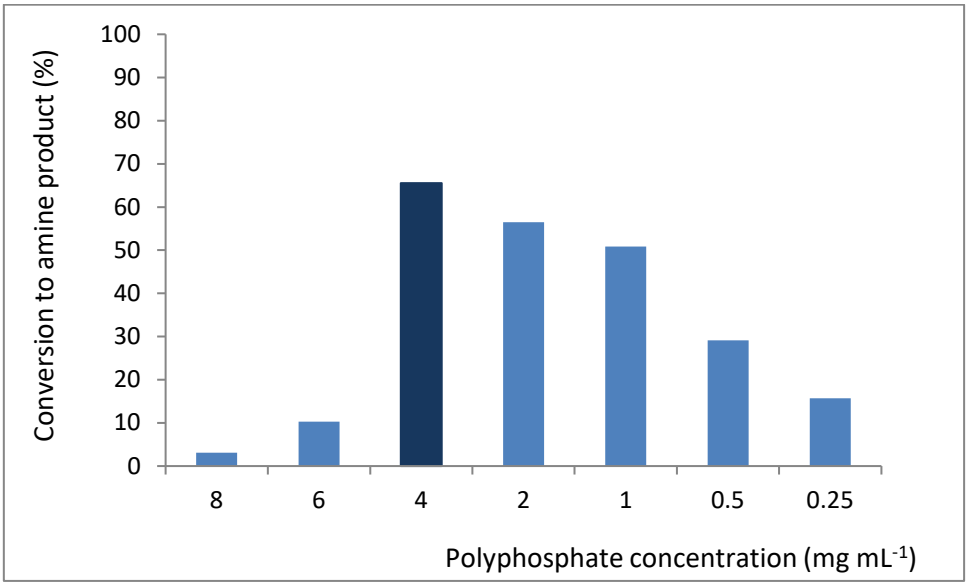


Figure S4 Polyphosphate loading. Reaction conditions: 5 mM dodecanoic acid, 50 mM $MgCl_2$, 50 mM D-glucose, 0.5 mM NADP+, 250 mM IPA, 1 mg mL^{-1} pure *Mc*CAR, 1 mg mL^{-1} pure *Sp*-TA, 2 mg mL^{-1} PAP lysate, 2 mg mL^{-1} Adk lysate, 0.2 mg mL^{-1} CDX-901 GDH, 5% (v/v) DMSO, 100 mM Tris-HCl pH 8, 500 µL n-heptane (1:1 v/v to aqueous phase).

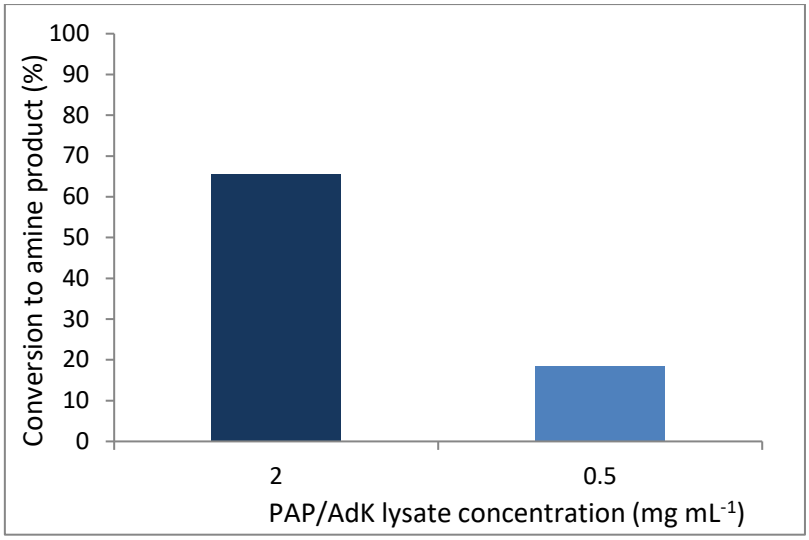


Figure S5 PAP/Adk lysate loading. Reaction conditions: 5 mM dodecanoic acid, 50 mM $MgCl_2$, 50 mM D-glucose, 4 mg mL^{-1} polyphosphate, 0.5 mM NADP+, 250 mM IPA, 1 mg mL^{-1} pure *Mc*CAR, 1 mg mL^{-1} pure *Sp*-TA, 0.2 mg mL^{-1}CDX-901 GDH, 5% (v/v) DMSO, 100 mM Tris-HCl pH 8, 500 µL n-heptane (1:1 v/v to aqueous phase).

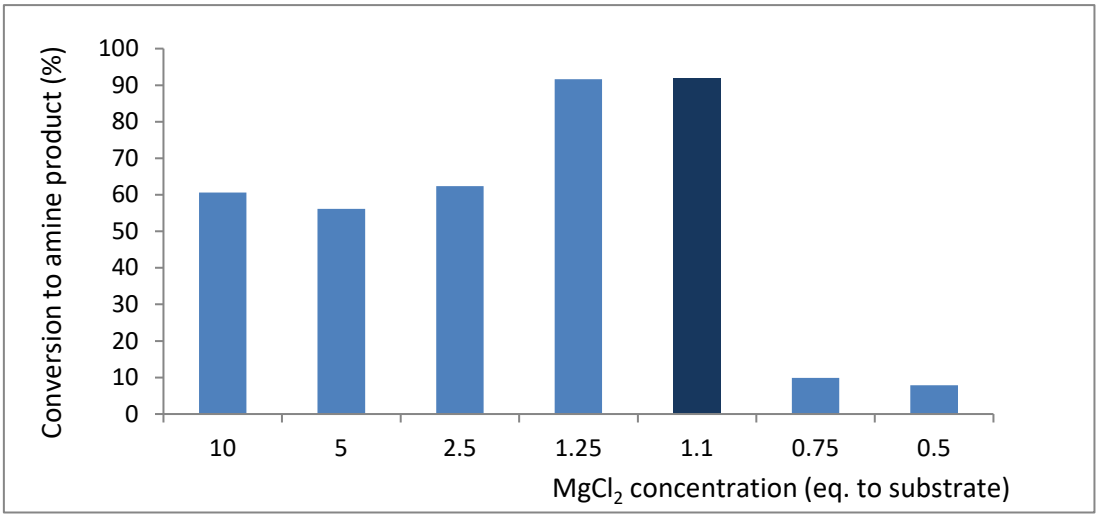


Figure S6 $MgCl_2$ loading. Reaction conditions: 5 mM dodecanoic acid, 50 mM D-glucose, 4 mg mL^{-1} polyphosphate, 0.5 mM NADP+, 250 mM IPA, 1 mg mL^{-1} pure *Mc*CAR, 1 mg mL^{-1} pure *Sp*-TA, 2 mg mL^{-1} PAP lysate, 2 mg mL^{-1} Adk lysate, 0.2 mg mL^{-1} CDX-901 GDH, 5% (v/v) DMSO, 100 mM Tris-HCl pH 8, 500 µL n-heptane (1:1 v/v to aqueous phase).

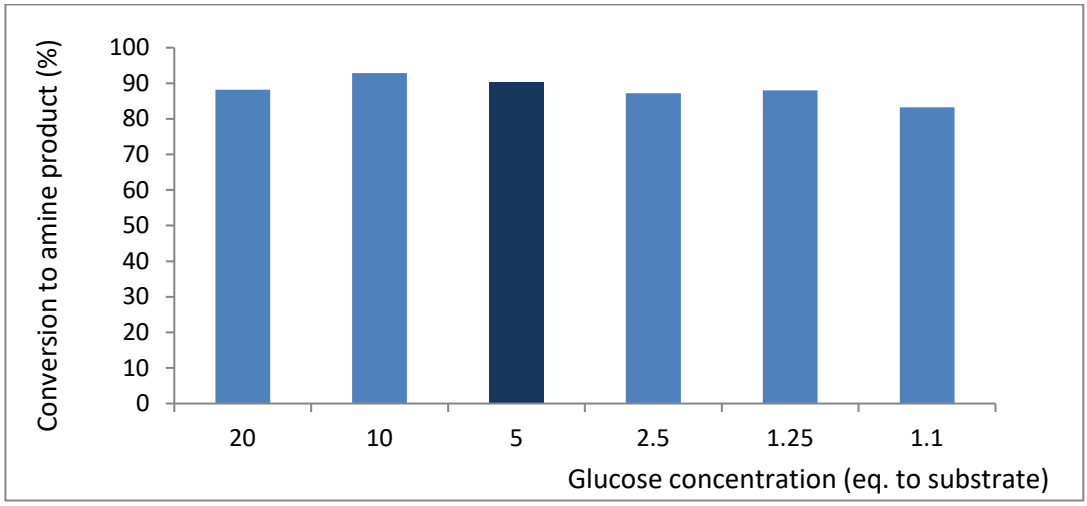


Figure S7 Glucose loading. Reaction aqueous phase conditions: 5 mM dodecanoic acid, 5.5 mM $MgCl_2$, 4 mg mL^{-1} polyphosphate, 0.5 mM NADP+, 250 mM IPA, 1 mg mL^{-1} pure *Mc*CAR, 1 mg mL^{-1} pure *Sp*-TA, 2 mg mL^{-1} PAP lysate, 2 mg mL^{-1} Adk lysate, 0.2 mg mL^{-1} CDX-901 GDH, 5% (v/v) DMSO, 100 mM Tris-HCl pH 8, 500µL n-heptane (1:1 v/v to aqueous phase).

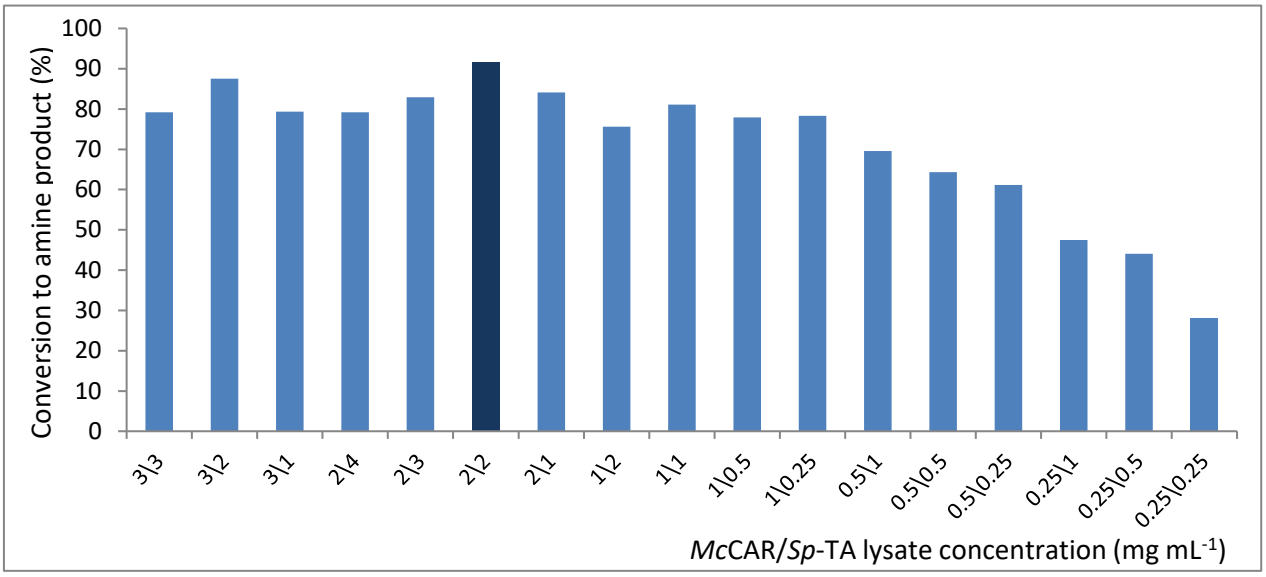


Figure S8 *Mc*CAR/*Sp*-TA lysate loading. Reaction conditions: 5 mM dodecanoic acid, 5.5 mM $MgCl_2$, 25 mM D-glucose, 4 mg mL^{-1} polyphosphate, 0.5 mM NADP+, 250 mM IPA, 2 mg mL^{-1} PAP lysate, 2 mg mL^{-1} Adk lysate, 0.2 mg mL^{-1} CDX-901 GDH, 5% (v/v) DMSO, 100 mM Tris-HCl pH 8, 500µL n-heptane (1:1 v/v to aqueous phase).

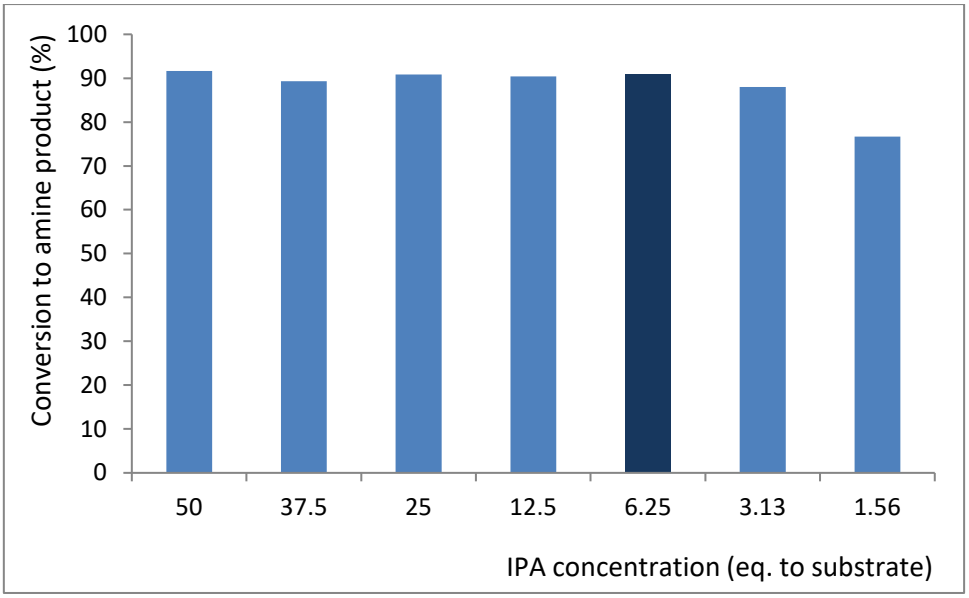


Figure S9 IPA loading. Reaction conditions: 5 mM dodecanoic acid, 5.5 mM $MgCl_2$, 25 mM D-glucose, 4 *mg* mL^{-1} polyphosphate, 0.5 mM NADP+, 2 *mg* mL^{-1} *Mc*CAR lysate, 2 *mg* mL^{-1} *Sp*-TA lysate, 2 *mg* mL^{-1} PAP lysate, 2 *mg* mL^{-1} Adk lysate, 0.2 *mg* mL^{-1} CDX-901 GDH, 5% (v/v) DMSO, 100 mM Tris-HCl pH 8, 500µL n-heptane (1:1 v/v to aqueous phase).

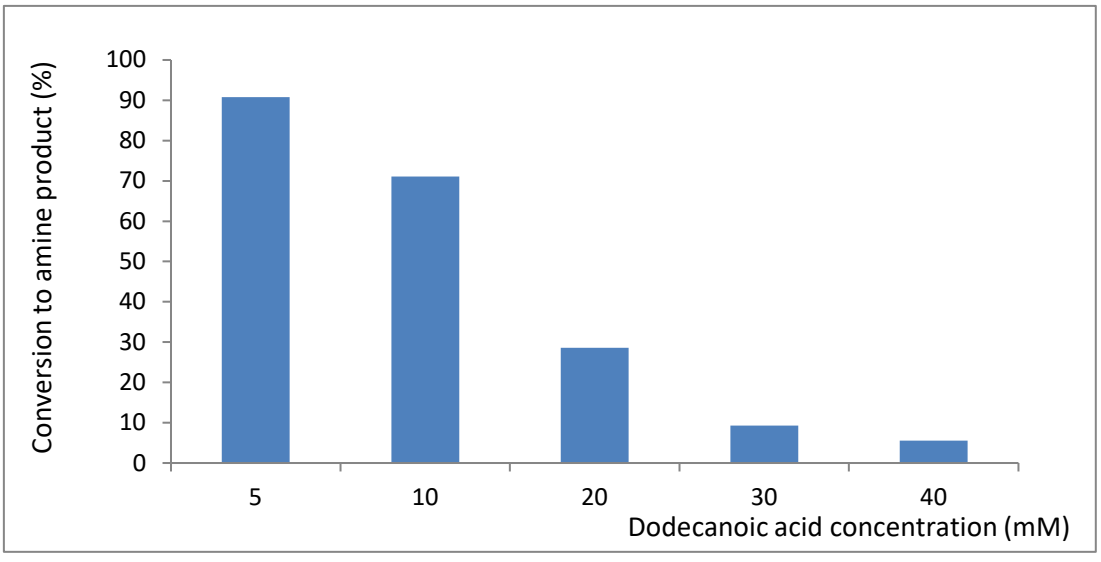
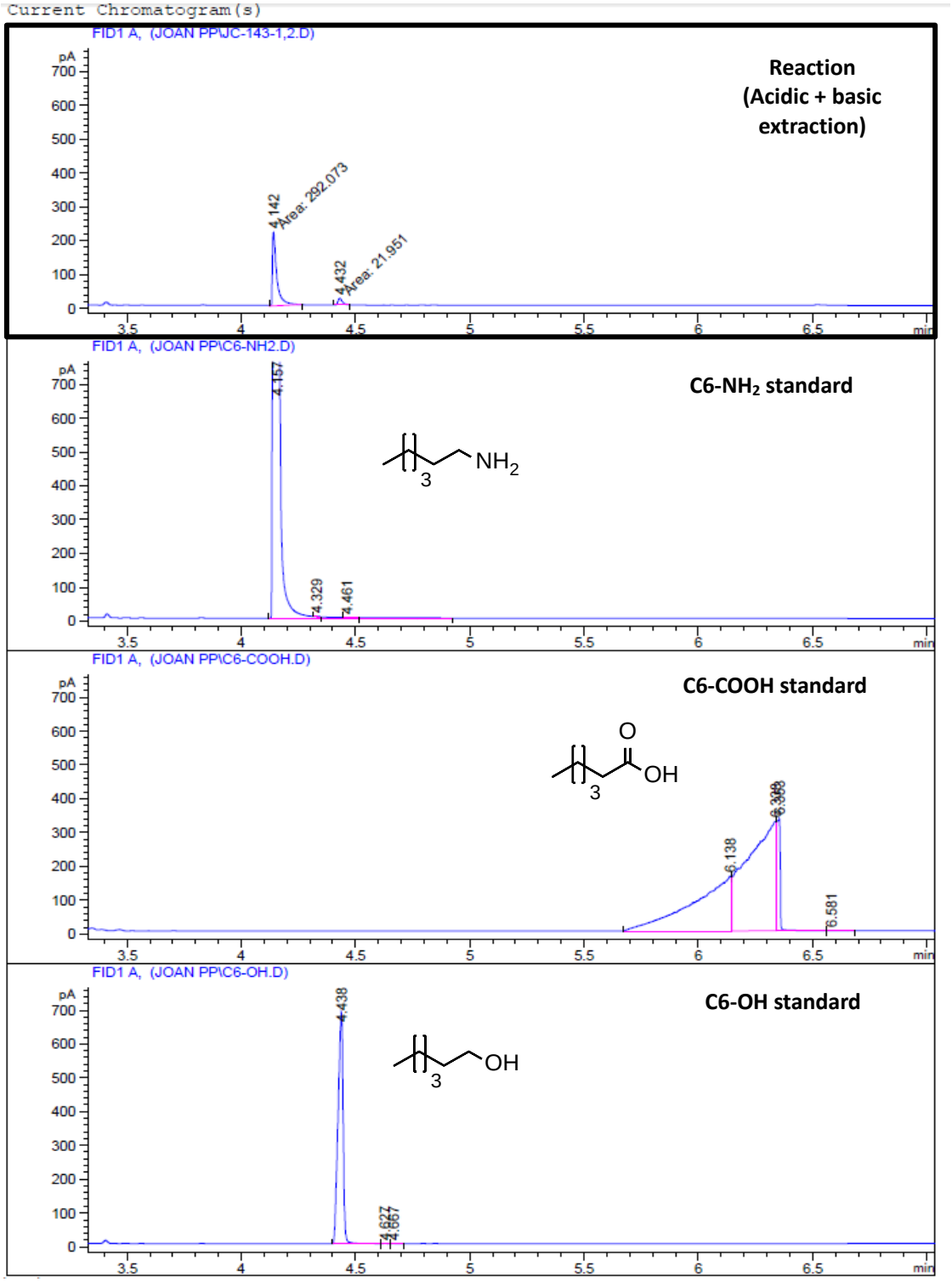


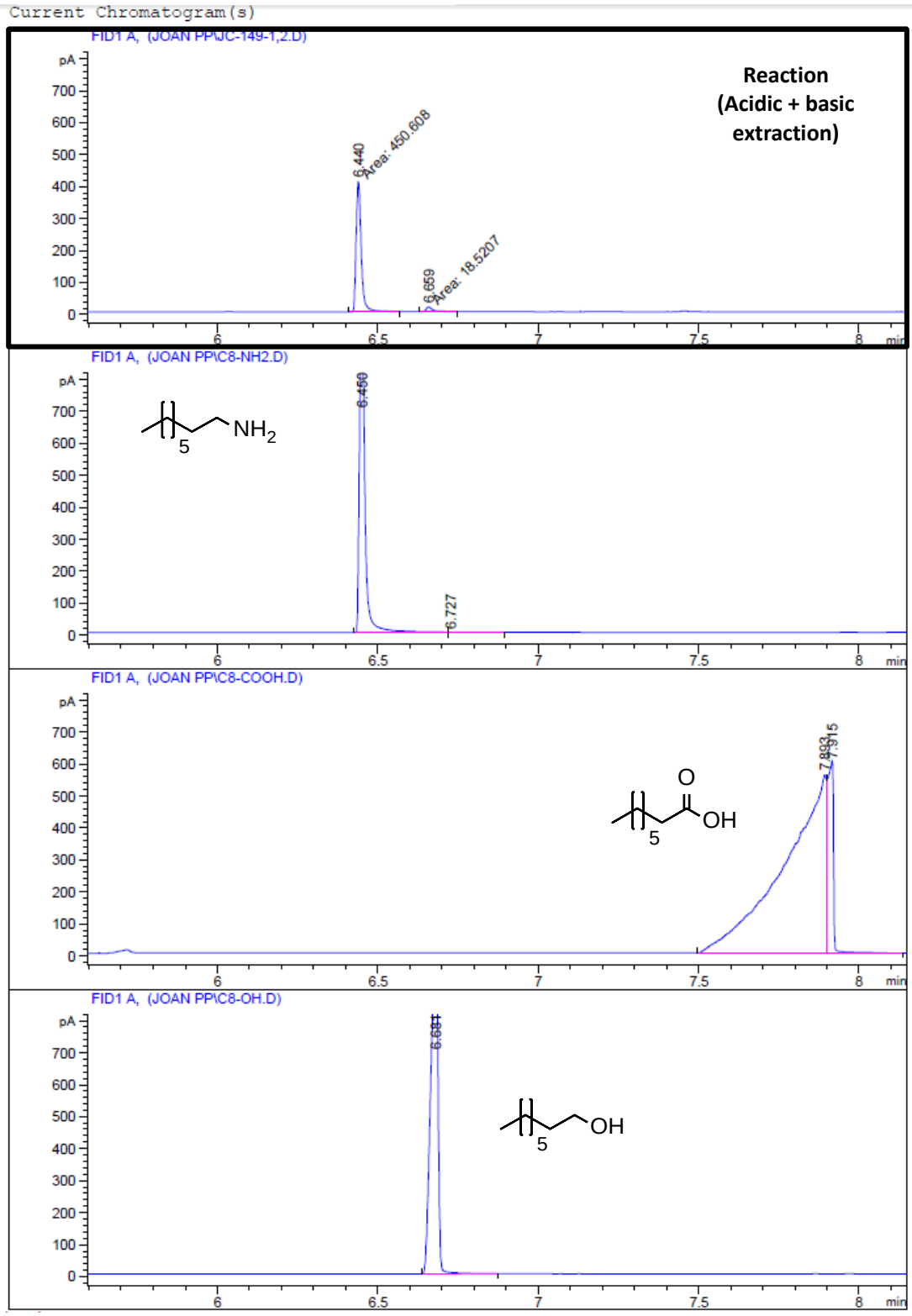
Figure S10 Substrate loading. Reaction conditions:, 5.5 mM $MgCl_2$, 25 mM D-glucose, 4 *mg* mL^{-1} polyphosphate, 0.5 mM NADP+, 2 *mg* mL^{-1} *Mc*CAR lysate, 2 *mg* mL^{-1} *Sp*-TA lysate, 2 *mg* mL^{-1} PAP lysate, 2 *mg* mL^{-1} Adk lysate, 0.2 *mg* mL^{-1} CDX-901 GDH, 5% (v/v) DMSO, 100 mM Tris-HCl pH 8, 500µL n-heptane (1:1 v/v to aqueous phase). *Concentrations were directly increased together with the substrate load to assure the same number of equivalents in all the reactions.

GC-FID and GC-MS traces for quantification and identification

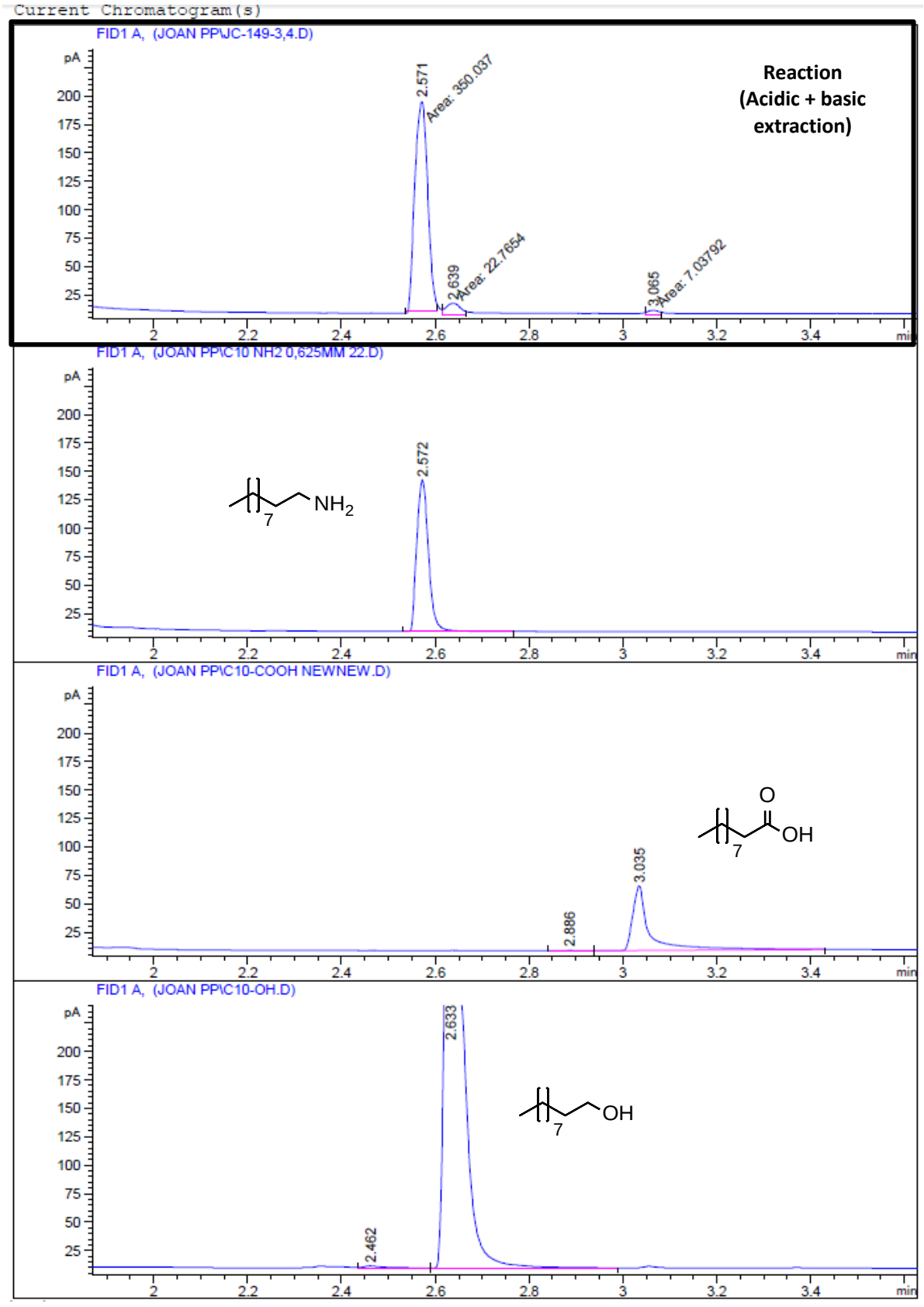
Hexanoic acid **1a**



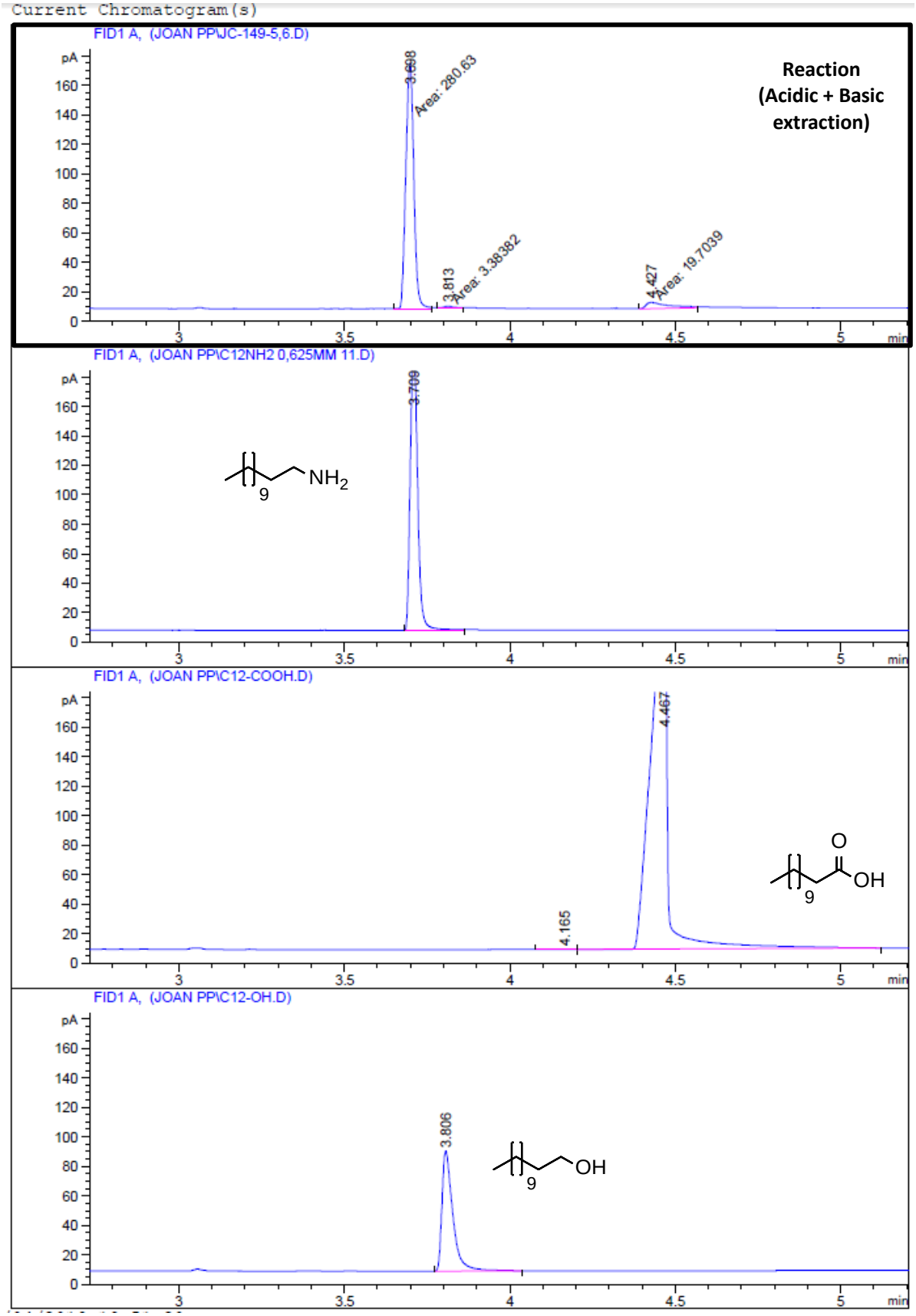
Octanoic acid **1b**



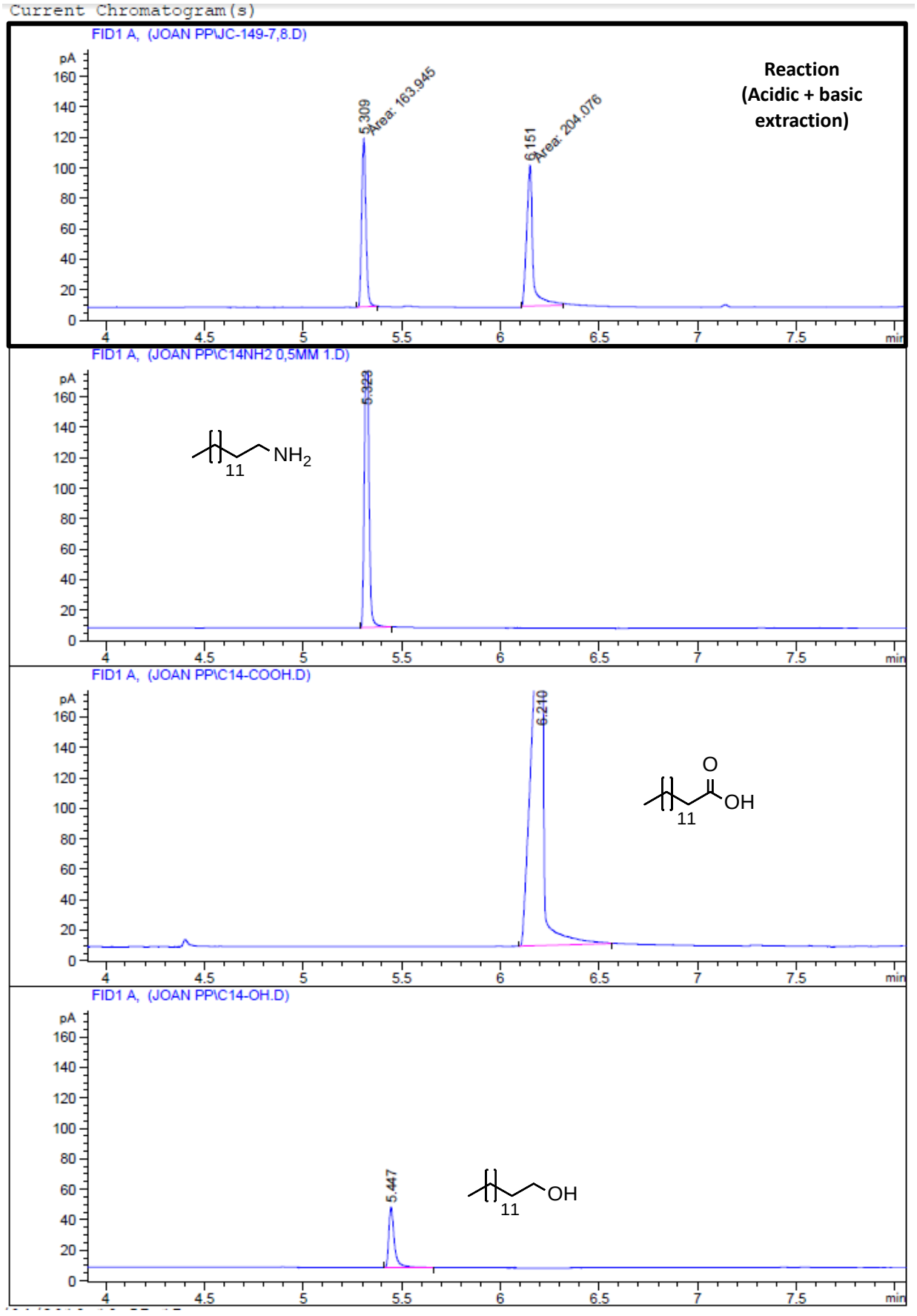
Decanoic acid **1c**



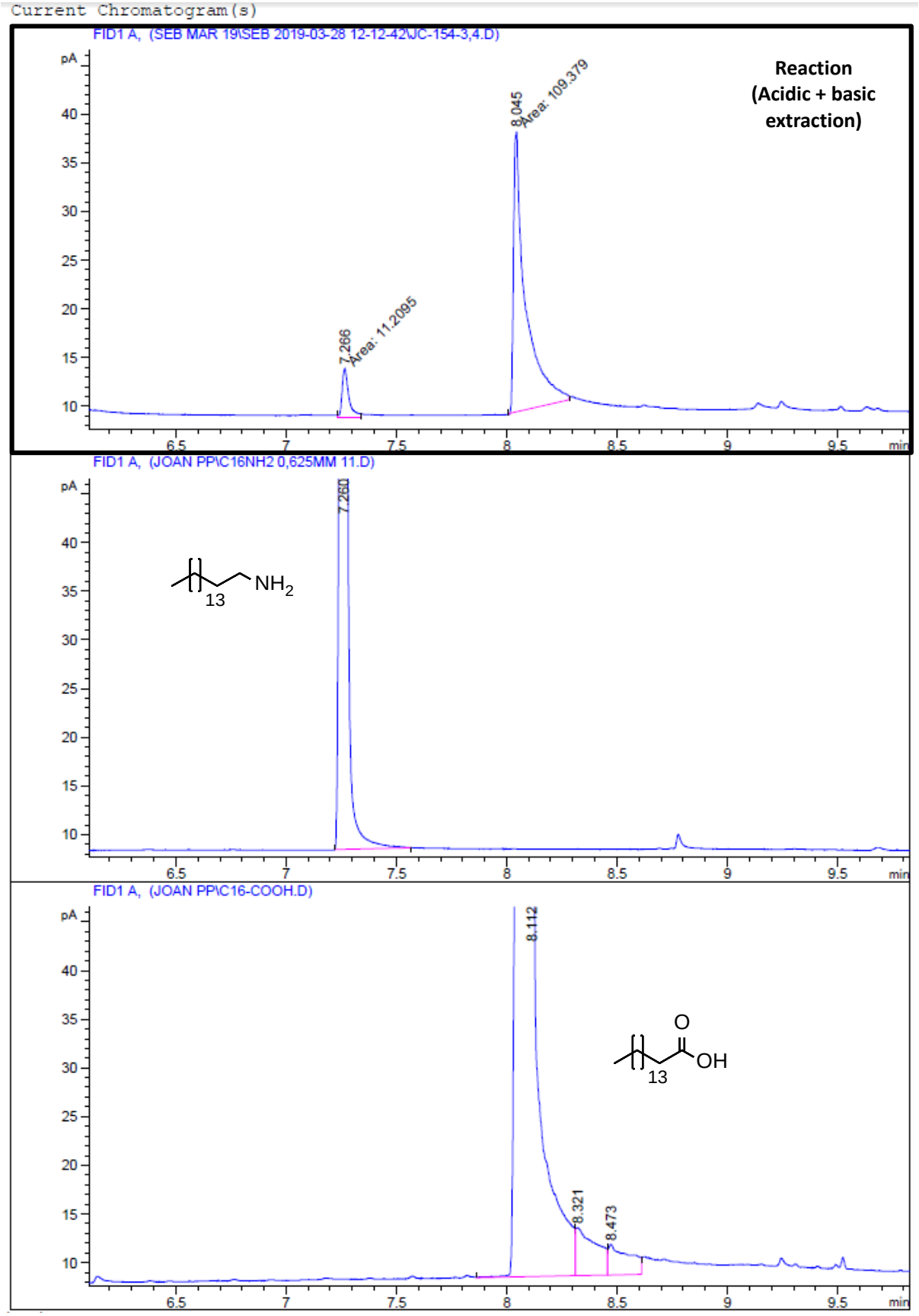
Dodecanoic acid **1d**



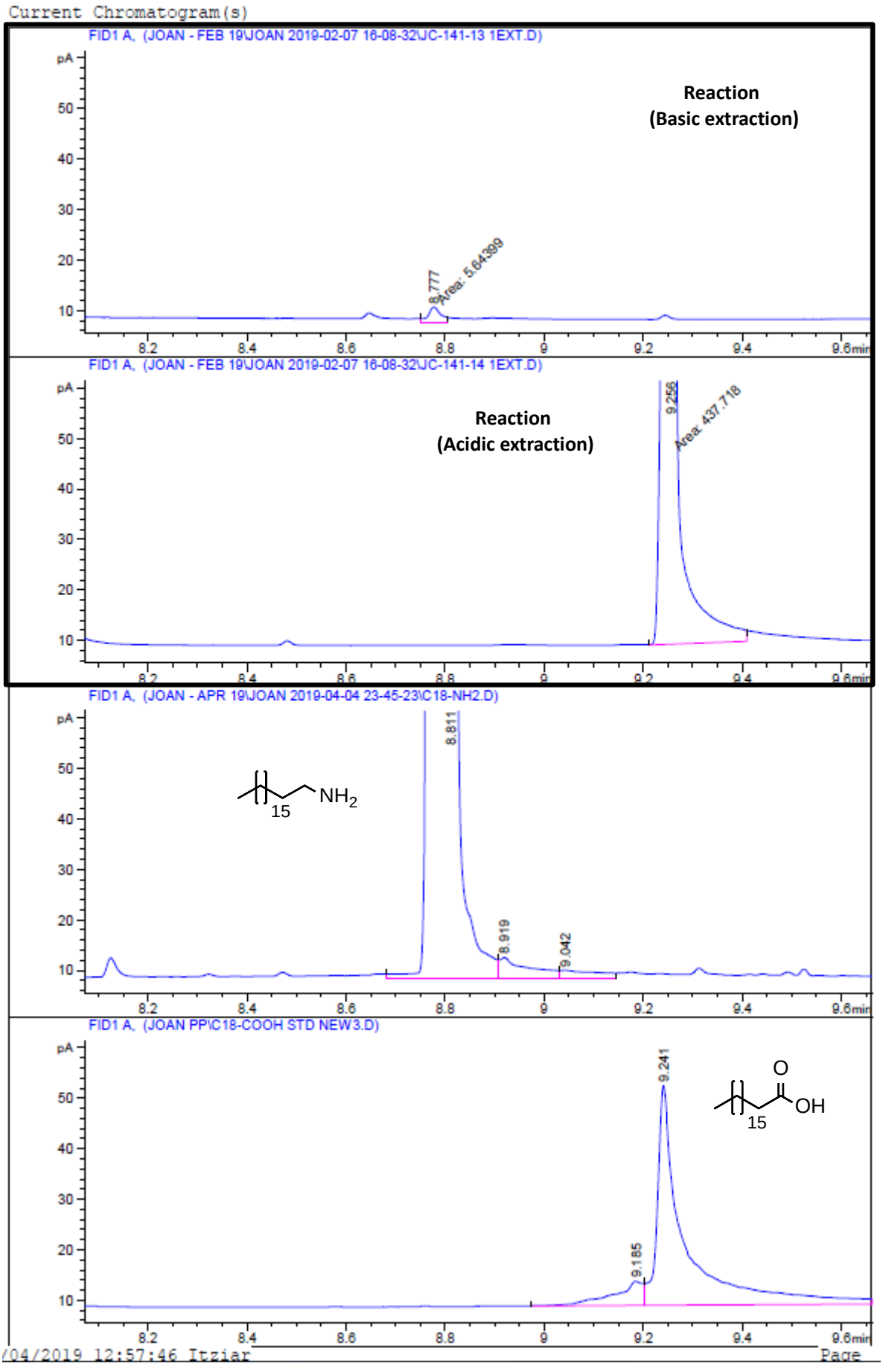
Myristic acid **1e**



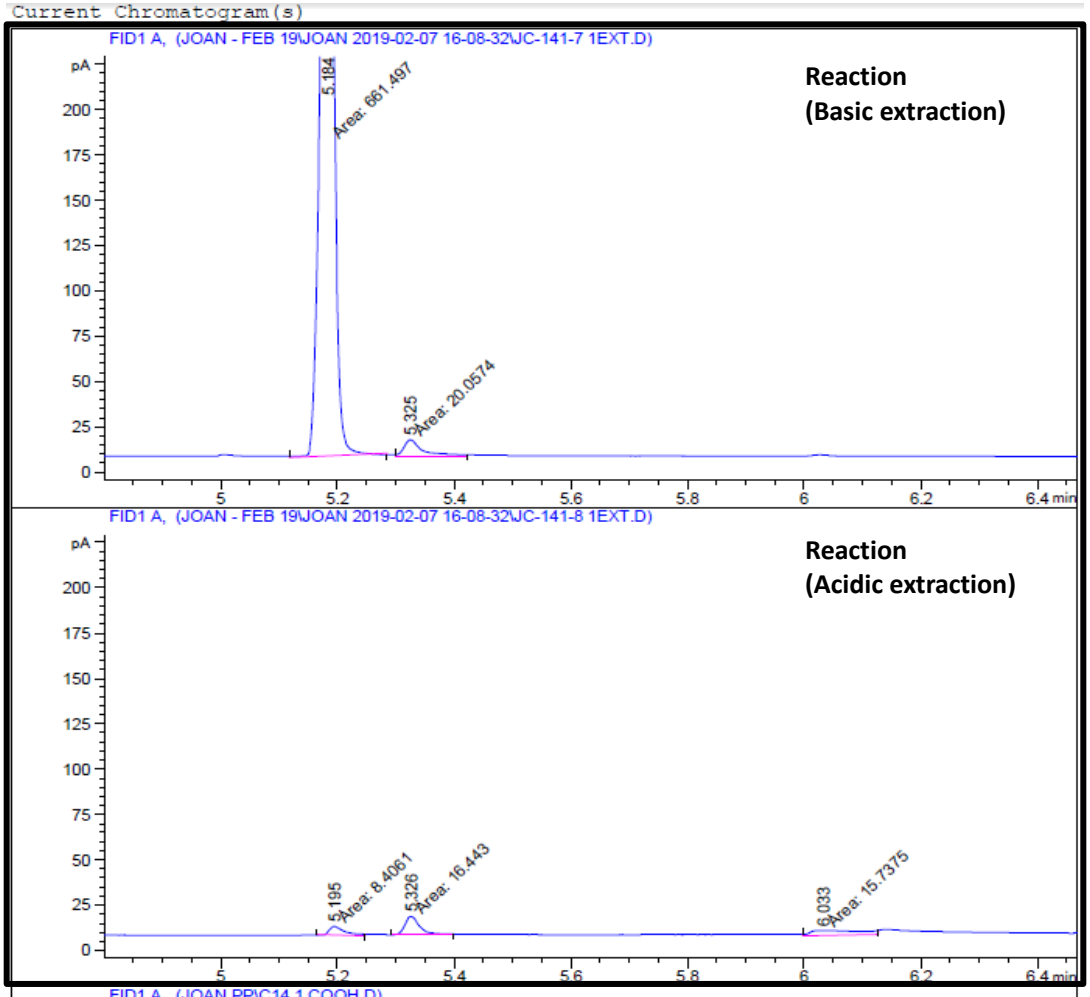
Palmitic acid **1f**

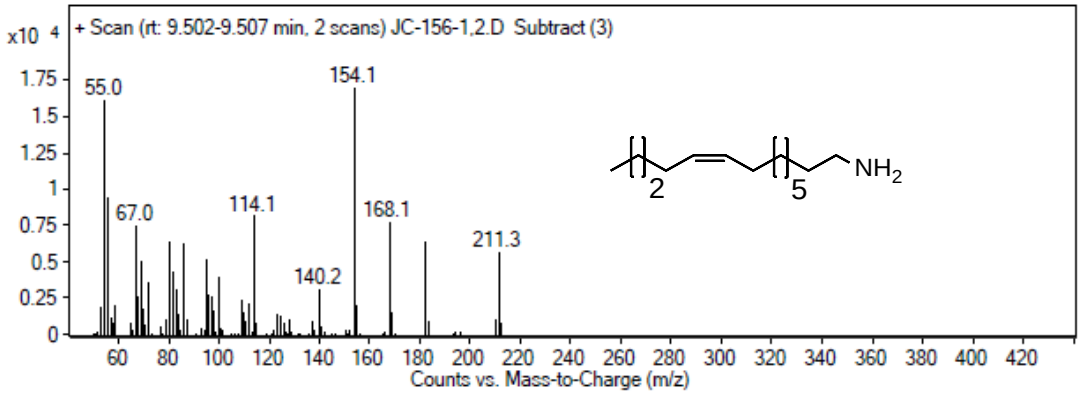


Stearic acid **1g**

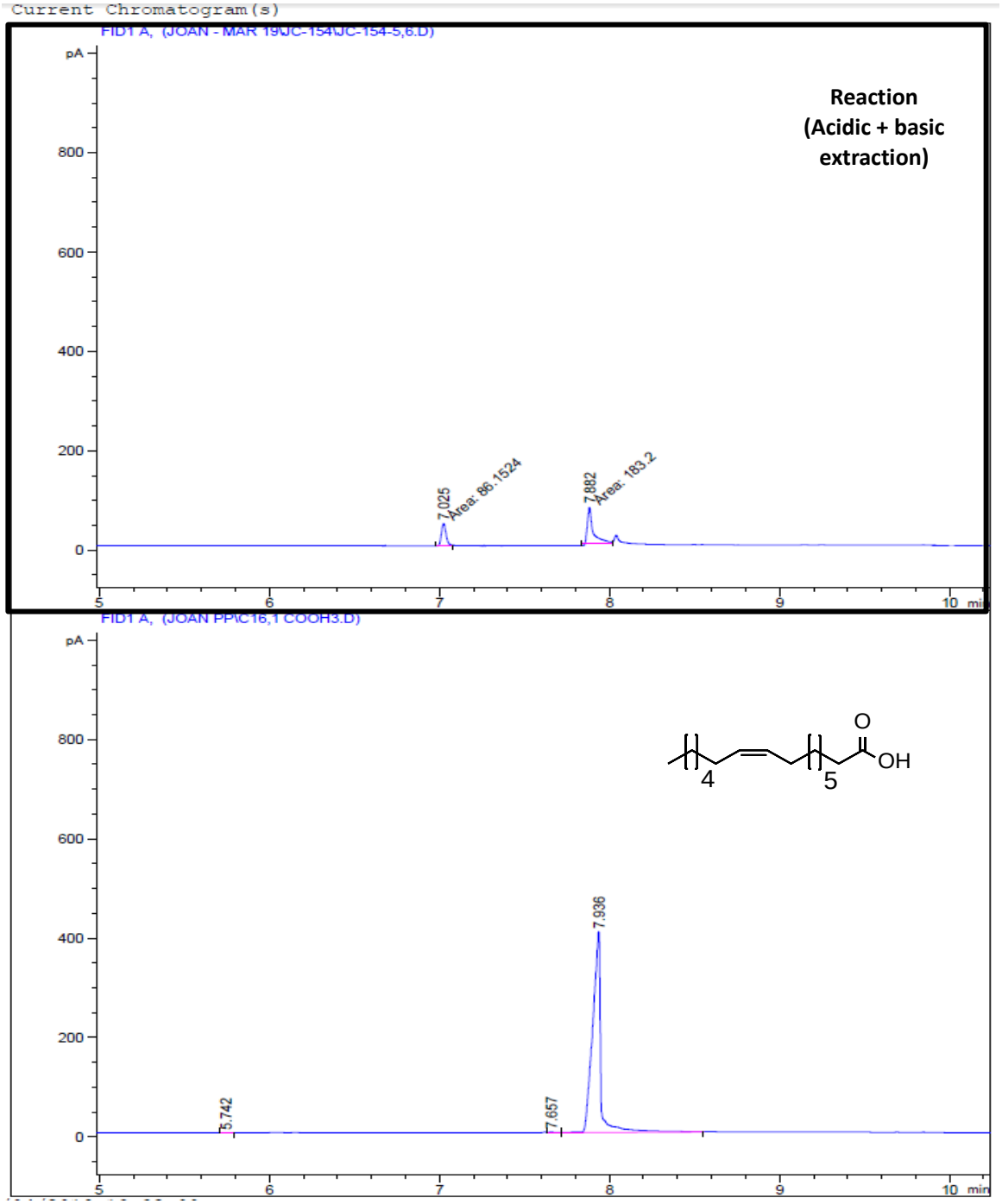


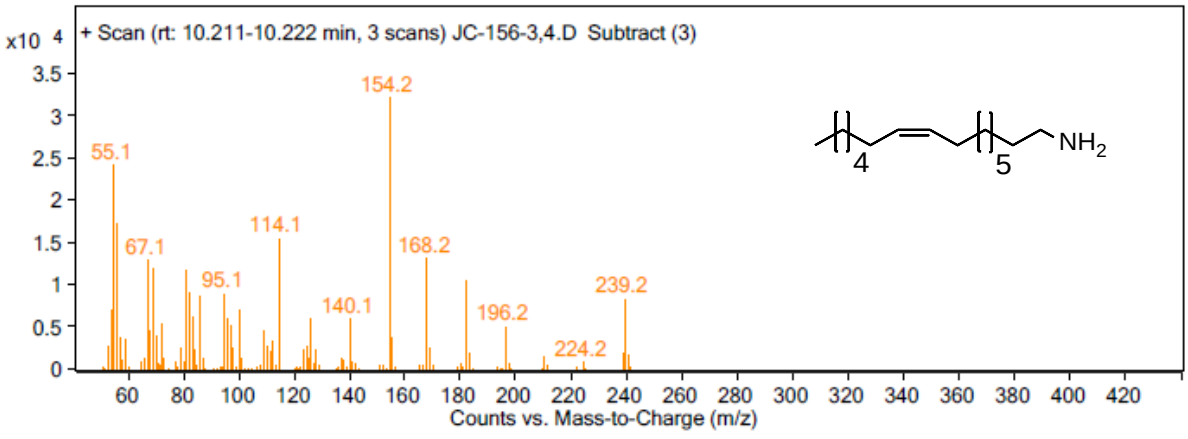
Myristoleic acid **1h**



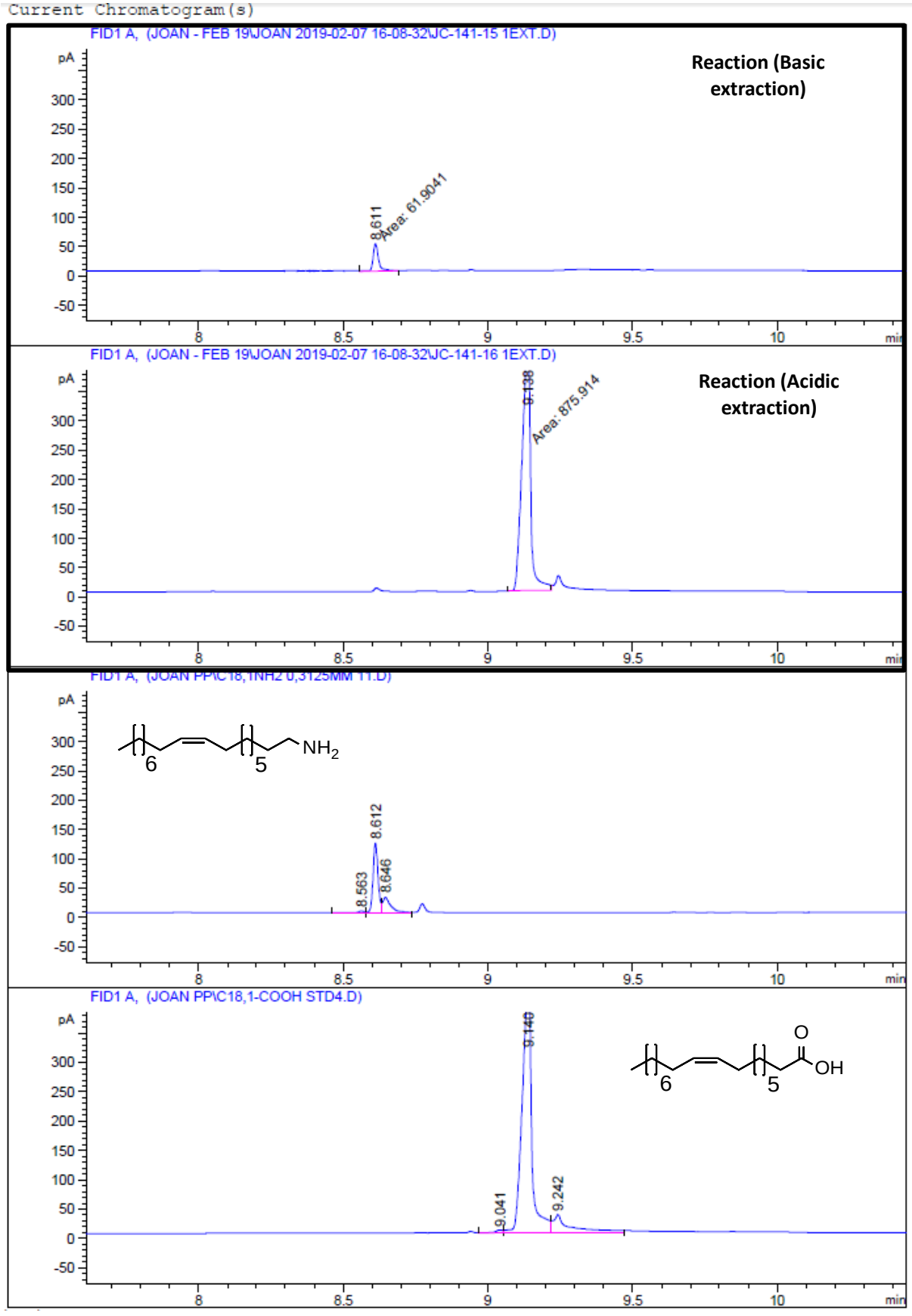


Palmitoleic acid **1i**

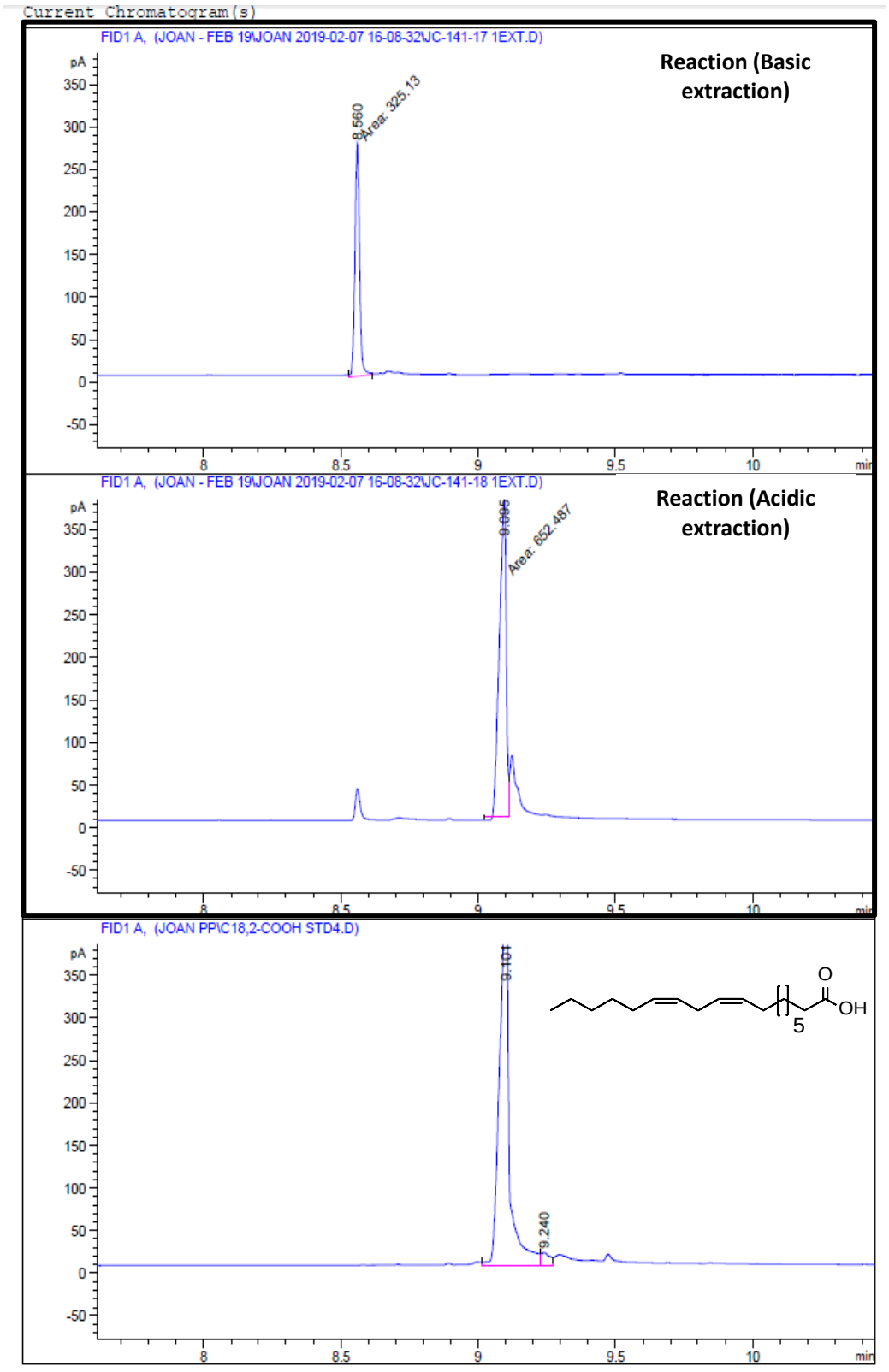




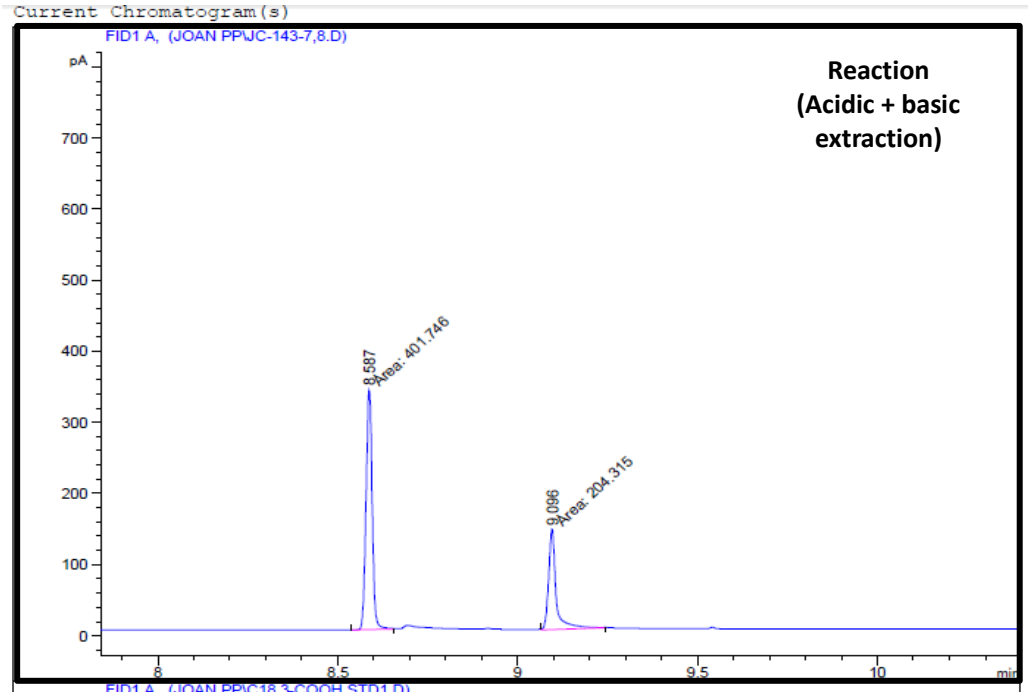
Oleic acid **1j**

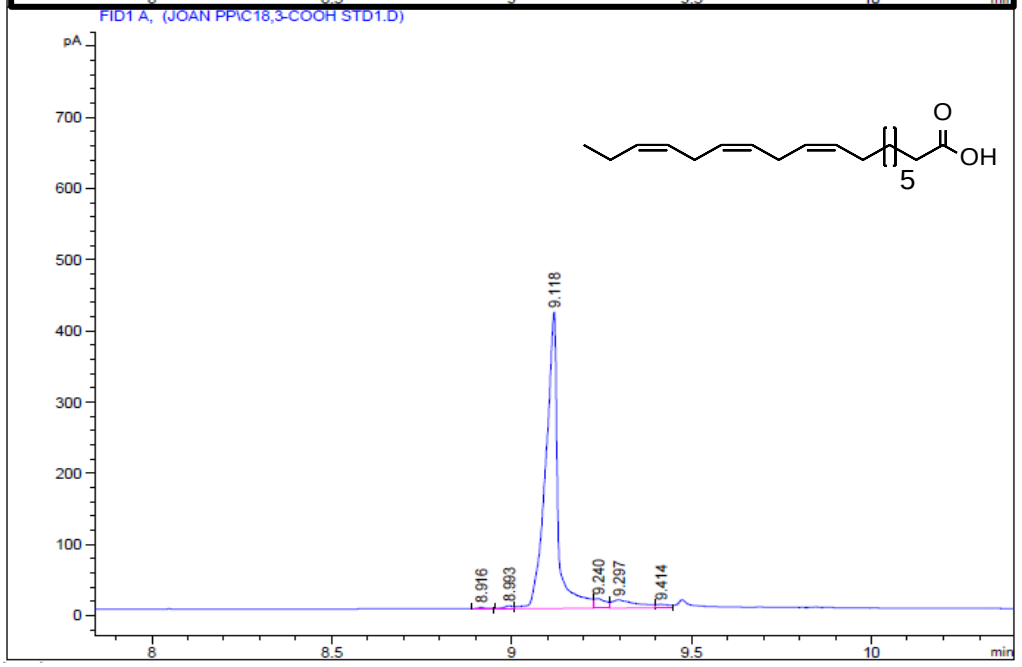


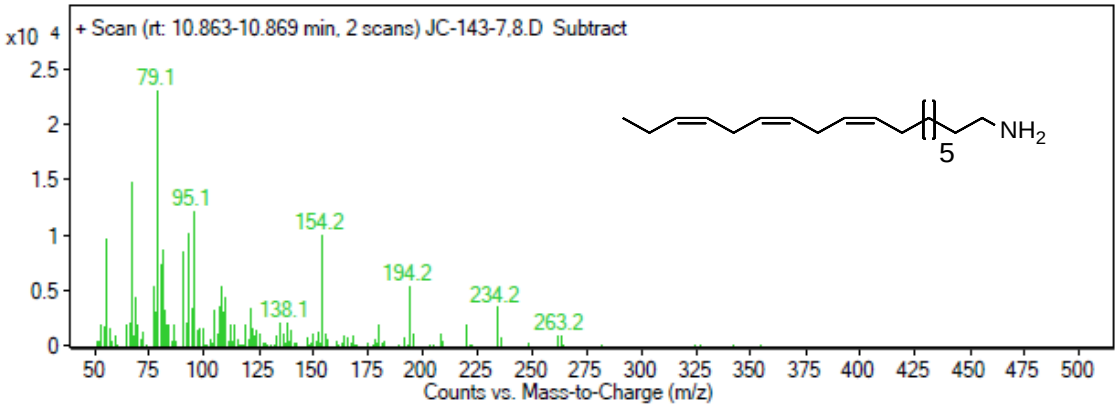
Linoleic acid **1k**



Linolenic acid **1l**







Specific activity assay

Specific activity of *Mc*CAR towards substrates **1d**, **1e** and **1h** was determined by spectrophotometric assay measuring the depletion of NADPH at 340 nm (ε = 6.6 mM cm^{-1}). Reactions were performed in triplicate in 200 µL of Tris-HCl pH 8. Reactions were performed in 100 mM Tris-HCl buffer pH 8 and measurements were taken every 5 seconds during 30 min.

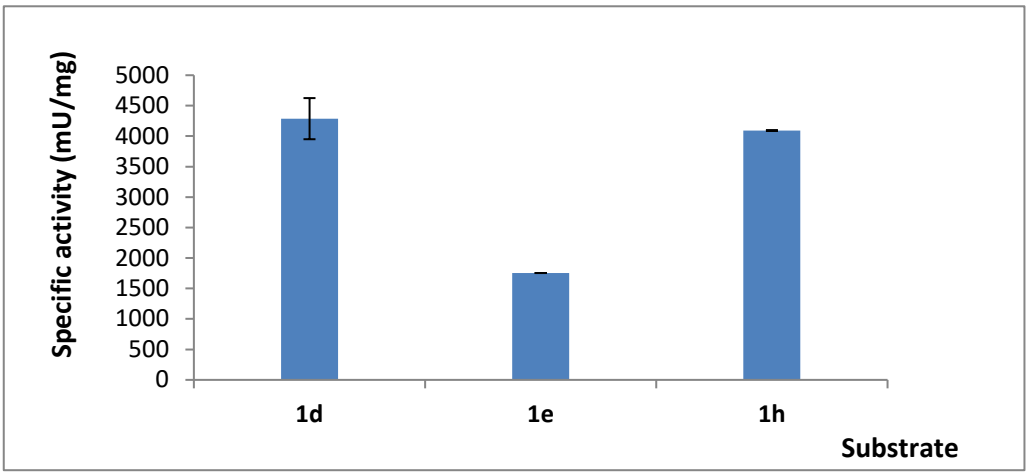
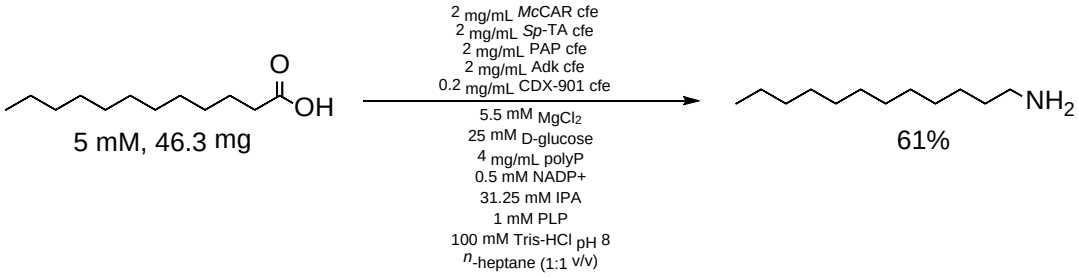


Figure S11
Specific activity assay. Reaction conditions: 0.1 mM substrate, 10 mM $MgCl_2$, 0.25 mM NADPH, 1 mM ATP, 6 µg pure *Mc*CAR, 5% (v/v) DMSO, 100 mM Tris-HCl pH 8.

Preparative-Scale biocatalytic amination of dodecanoic acid



For the reaction preparation, 100mM Tris-HCl pH 8 (containing 1 mM PLP) was used to dissolve 225 mg of D-glucose, 26 mg of $MgCl_2$, 21 mg of NADP+, 200mg of polyP, 1.57mL of 1 M IPA stock solution (in Tris-HCl pH 8), 10 mg of CDX-901 freeze-dried CFE, 1.1 mL of 92 mg/mL of *Mc*CAR CFE stock solution (in Tris-HCl pH 8), 1.4 mL of 70mg/mL *Sp*-TA CFE stock solution (in Tris-HCl pH 8), 1.2 mL of 85 mg/mL PAP CFE stock solution (in Tris-HCl pH 8) and 1.2 mL of 85 mg/mL Adk CFE stock solution (1.2 mL, 2 mg/mL) in a 500 mL round bottom glass bottle. The reaction was topped up to 50mL with 100mM Tris-HCl pH 8 (containing 1 mM PLP) and 50 mL of *n*-heptane were added on top to create a biphasic system (1:1 v/v between the aqueous and the organic phase). Dodecanoic acid was dissolved in DMSO to create a 0.1 M stock solution and 2.5 mL were added into the organic phase (46.3 mg). The reaction was incubated at 30 ºC and 80 rpm in an orbital shaker for 20 h. Reaction quenching and basification were performed by adding 10 M NaOH into the biphasic system to reach pH 12, followed by 50 mL of *n*-heptane making the aqueous phase/organic

phase ratio 1:2. The reaction was extracted 3 times with *n*-heptane (3 x 75mL) and centrifuged at 4000rpm for 10min to assure perfect phase separation. Combined organic layers were dried over $MgSO_4$, filtered and dried under vacuum yielding 28.2 mg of dodecylamine (0.153 mmol, 61%).

^{1}H NMR δ_H (400 MHz, CDCl3) 2.61 (t, *J* = 7.0 Hz, 2H), 1.42 – 1.30 (m, 2H), 1.28 – 1.11 (m, 20H), 0.81 (t, *J* = 6.7 Hz, 3H).
^{13}C NMR δ_C (100 MHz CDCl3) 42.31, 33.94, 31.93, 31.90, 29.69, 29.66, 29.64, 29.54, 29.37, 26.92, 22.70, 14.13;
HRMS calc. for C12H27N 186.22163 [M+H]+ , found 186.22245 (mass error, 4.403355 ppm).