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Supplementary Information

Continuous flow synthesis of the ionizable lipid ALC-0315

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Materials and Methods

Starting Materials

All reagents were used as received from commercial suppliers unless otherwise noted. Details on chemicals used:
1,6-Hexanediol (>97 %, 500 g, TCI Japan)
2-Hexyldecanoic acid (>98%, 500 g, TCI Japan)
4-Aminobutan-1-ol (> 98%, 25 g, TCI Japan,)
$NaHCO_3$ (≥ 99% Ph. Eur., 1 kg, Roth)
Na_2CO_3 (≥ 99% Ph. Eur., 1 kg, Roth)
Tetramethylammonium triacetoxyborohydride (95 %, 25 g, Sigma Aldrich)
Sodium hypochlorite pentahydrate (25 g, TCI Japan).
Dimethylformamide (water < 150 ppm, for synthesis, 2.5 l, VWR)
Thionylchloride (for synthesis, 1 L, Merck)
NaOH (≥ 98 %, p.a. ISO, Roth)
KBr (≥ 99 %, p.a. ACS, 500g, Roth)
TEMPO (98%, 25 g, Acros Organics)
1-Methylimidazole (1 L, GPR REACTAPUR®, VWR)
$Cu^{I}(MeCN)_4OTf$ (1 g, Sigma-Aldrich)
2,2'Bipyridine (25 g, TCI Japan)

Solvents

All solvents for chemical reactions were commercially purchased in p.a. quality unless otherwise noted. Solvents for flash chromatography were distilled prior to usage. Details on solvents used:
2-Methyltetrahydrofuran (anhydrous, stabilized, 10 L, Acros Organics)
Acetonitrile (≥ 99.9%, HPLC gradient grade, 2.5 L, Fisher)
1-Methylpyrrolidin-2-one (99%, extra pure, 2.5 L, Acros Organics)
Hexanes (mixture of isomers, technical, 200 L, VWR)
Ethyl acetate (technical, 200 L, VWR)
Methanol (Ph. Eur., EMPROVE® ESSENTIAL, 180 L, Merck)
For analytical chromatography, HPLC grade solvents were used.
All deuterated solvents were purchased from Carl Roth GmbH, $CDCl_3$ 99.8 Atom%D, d_8-toluene 99.5 Atom%D.

Analytical Methods

Chromatography

Flash column chromatography was performed on Kieselgel 60 with 230-400 mesh (Sigma-Aldrich, St. Louis, USA).
Analytical NP-HPLC was conducted on a Knauer Azura system, unless otherwise noted.

NMR Spectroscopy

NMR spectra were recorded on a Varian 400-NMR spectrometer operating at 400 MHz and 100.6 MHz for ^{1}H and ^{13}C respectively. Chemical shifts (δ) are reported in parts per million (ppm)

relative to the respective residual solvent peaks ($CDCl_3$: δ 7.26 in ^1H NMR spectra and δ 77.16 in ^{13}C NMR spectra; toluene-d_8 δ 2.08 in ^1H and 137.48 in ^{13}C). The following abbreviations are used to indicate peak multiplicities: s (singlet), d (doublet) dd (doublet of doublets), t (triplet), dt (doublet of triplets), td (triplet of doublets), q(quartet), p (pentet), m (multiplet). Coupling constants (J) are reported in Hertz (Hz). NMR spectra were processed using MestreNova 14.1 (MestreLab Research).

Mass Spectrometry

High resolution mass spectra (HRMS) were obtained using 6210 ESI-TOF mass spectrometer (Agilent).

FT-IR

Inline FT-IR measurements were acquired with a Flow-IR spectrometer (Mettler-Toledo, Columbus, USA).

Flow Equipment

Pumps

For delivering chemical feeds, HPLC pumps with 10 mL stainless steel pumpheads (Compact pump, Knauer GmbH, Berlin, Germany) or continuous syringe pumps Microlab 600 (Hamilton Bonaduz AG, Bonaduz, Switzerland) were used.

Reaction Temperature Control

Heating and cooling of reactions was provided by a Vapourtec R4 heater (Vapourtec Ltd., Bury St Edmunds, UK) unless otherwise noted.
Room temperature (RT) is reported as 25 °C

Pressure control

For pressurization of reactors, backpressure regulators of specified rating were used (e.g. P-787, IDEX HS).

Mixing

For mixing, T-mixers (PEEK Low Pressure Tee Assembly 1/16" PEEK .040 thru hole, P-714, IDEX HS) and Cross-mixers (P-634 ETFE Cross assembly for 1/16” OD Tubing 1/4-28 Flat-Bottom 0.020” through hole P-245, IDEX HS) were used.

Tubing

ETFE tubing (1 mm ID, 1/16” OD) and PTFE tubing (1 mm ID, 1/16” OD) for flow experiments was purchased from IDEX HS.

Mass Flow Controller

O_2 thermal Mass Flow Controller, 25 ml·min^{-1} (SLA5850SC1CF1B2A1, Brooks, USA)

Experimental Procedures

Schematic representation of the overall flow synthesis setup

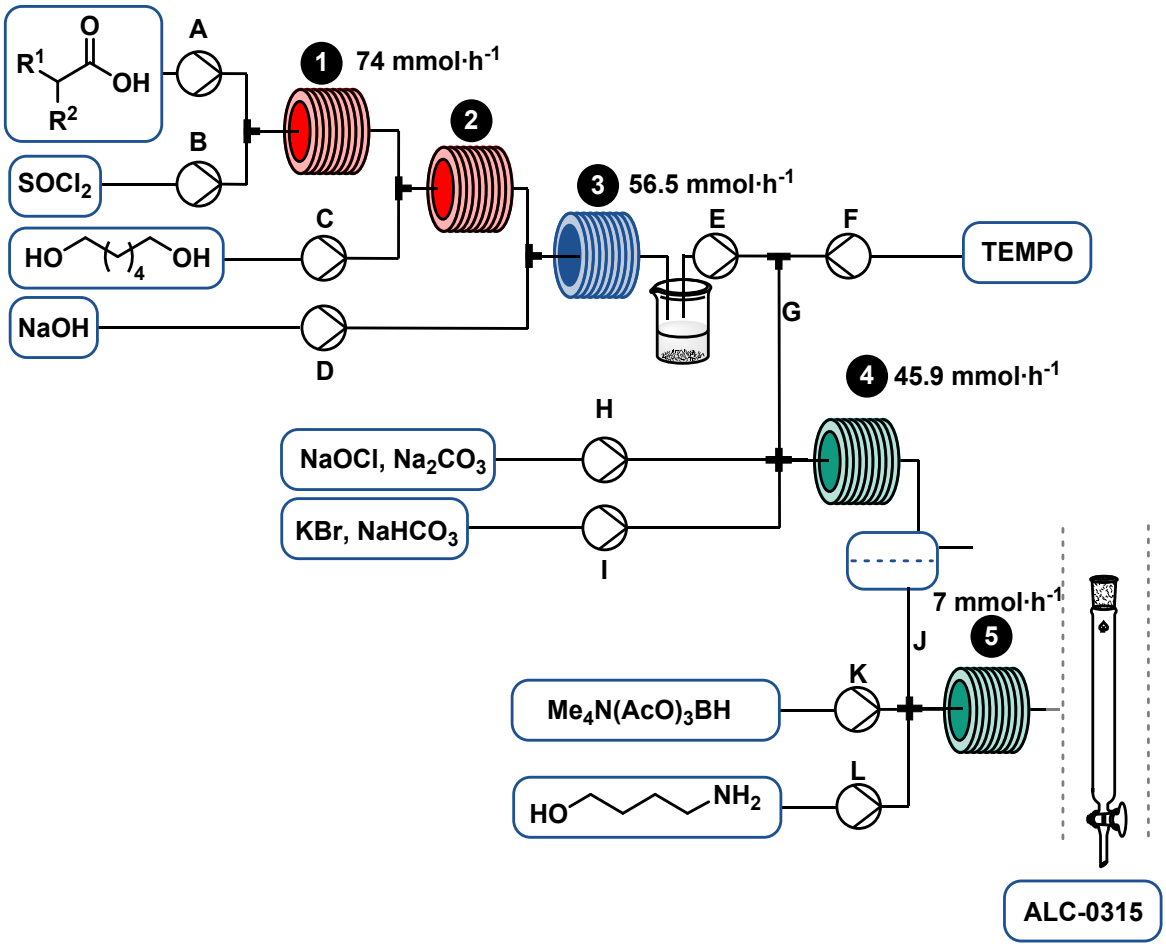
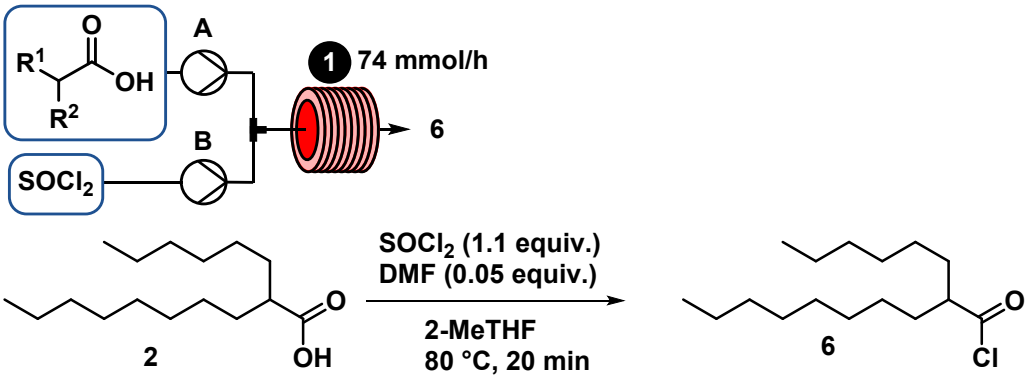


Figure 1: Flow scheme for the reported synthesis of ALC-0315.

2-hexyldecanoyl chloride (**6**)



A solution of **2** (35.23 g, 137.4 mmol, 1.0 equiv.) and *N*,*N*-dimethylformamide (502 mg, 6.87 mmol, 5 mol%) in 2-methyltetrahydrofuran (1.374 M) was pumped through 1/16" ETFE tubing (I.D. 1 mm) using an HPLC pump at a flow rate of

0.901 mL·min-1 [Line A]. Neat thionyl chloride (18.00 g, 151.3 mmol) was pumped through ETFE tubing (I.D. 1 mm) using a continuous syringe pump at a flow rate of 0.099 mL·min^{-1} [Line B]. Line B was combined with Line A via a T-mixer providing a continuous flow of a solution, which was heated at 80 °C in a 20 mL reactor (Reactor 1, 20 min residence time, ETFE tubing).
An analytical sample of **6** was obtained by removing the solvent under reduced pressure, yielding a pale yellow oil.
^{1}H NMR (400 MHz, $CDCl_3$) δ = 2.78-2.71 (m, 1H), 1.76-1.69 (m, 2H), 1.56-1.49 (m, 2H), 1.39-1.18 (m, 20H), 0.9-0.86 (m, 6H).
IR 1797 cm^{-1} (R-C=OCl) 1738 cm^{-1} R-C=OOH

In order to optimize time and temperature for acid activation, we set the concentration of carboxylic acid to 1.374 M to arrive at a 1:9 ratio of flow rates with 1.1 equiv. of thionyl chloride. To speed up the reaction, we added 2.5 mol% of DMF as a catalyst. At steady-state conditions (two reactor volumes), we determined the presence or absence of the carbonyl stretch in the IR spectrum as a proxy for full conversion as a function of temperature (residence time = 5 min). No full conversion was obtained at 80 °C or lower after 5 min. At 100 °C, the reactor outlet turned brownish-black, which we took as a sign of degradation. We therefore settled to 80 °C as maximum temperature and increased the residence time, until no signal of the carbonyl stretch of the acid was visible anymore in inline IR. Since we observed reactor fouling subsequently, due to small amounts of free carboxylic acid remaining in the telescoped process, we submitted the crude acid chloride solution to NMR analysis, which revealed the presence of low amounts of free carboxylic acid. In order to speed up the reaction without changing other parameters, we therefore doubled the amount of catalyst (DMF). Subsequent analysis based on NMR of the crude reactor outlet did not show any signal of remaining acid. Additional complications of bespoke optimization were the corrosion of downstream back-pressure regulators needed to achieve superheating.

Note on acyl chloride formation:
When telescoping these first two steps, we observed a decrease in backpressure over time – upon examination of the back pressure regulator (BPR) cartridge (2.8 bar, IDEX) the gold-plated spring had dissolved. We found that a combination of thionyl chloride and DMF is known from literature to dissolve noble metals.[1]

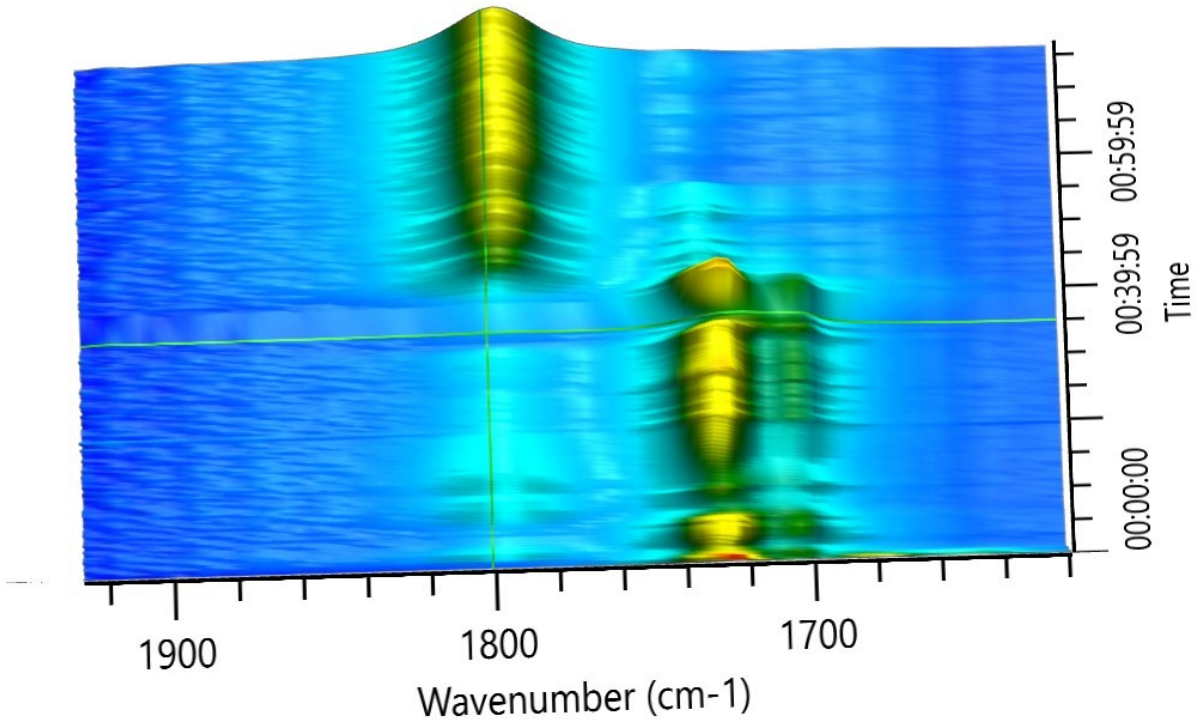


Figure 2: Inline IR measurements at the acyl chloride reactor outlet. Disappearance of the peak at lower wavenumber (1738 cm^{-1}) indicates full conversion of the carboxylic acid. Intensity is color-coded.

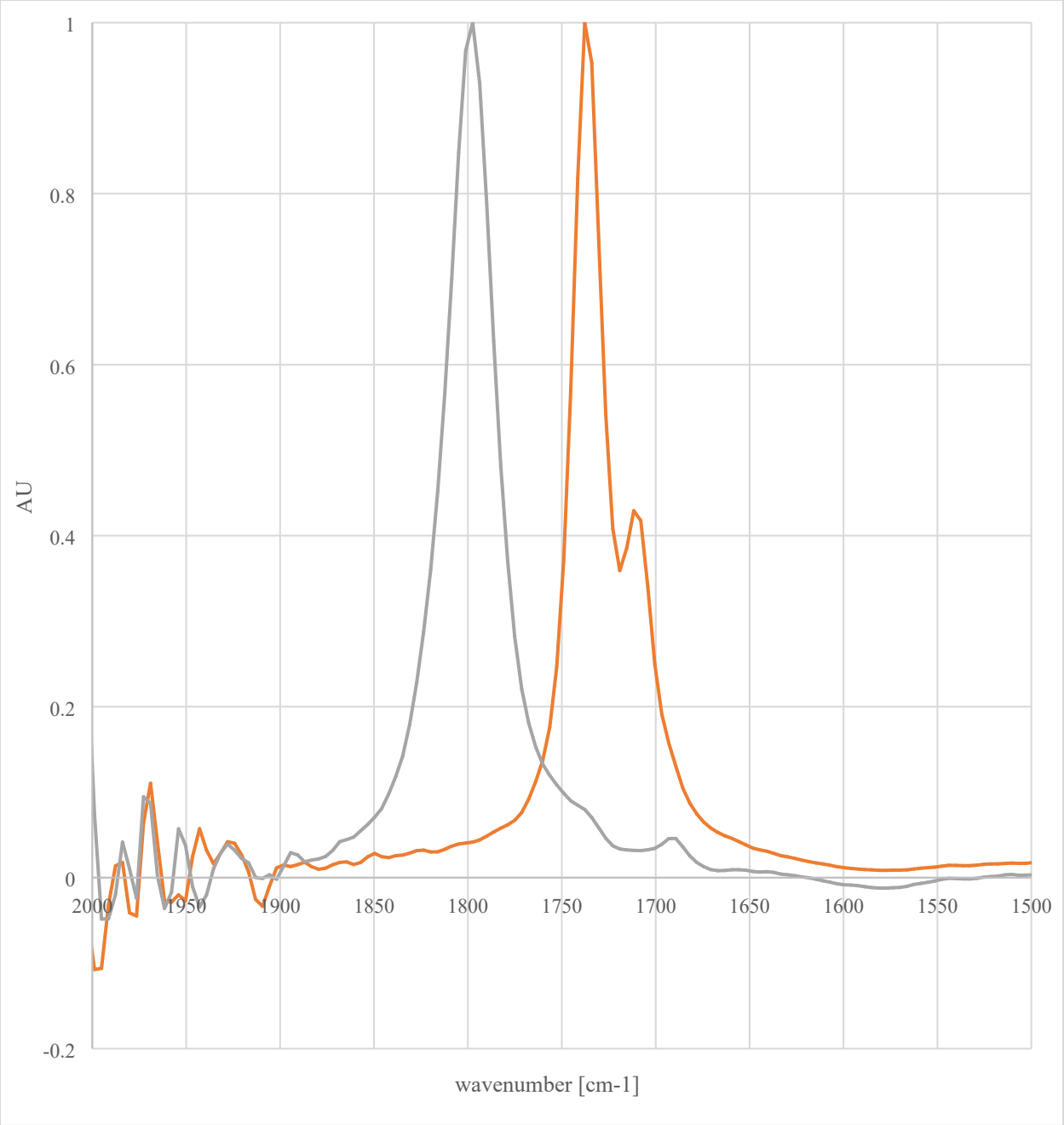
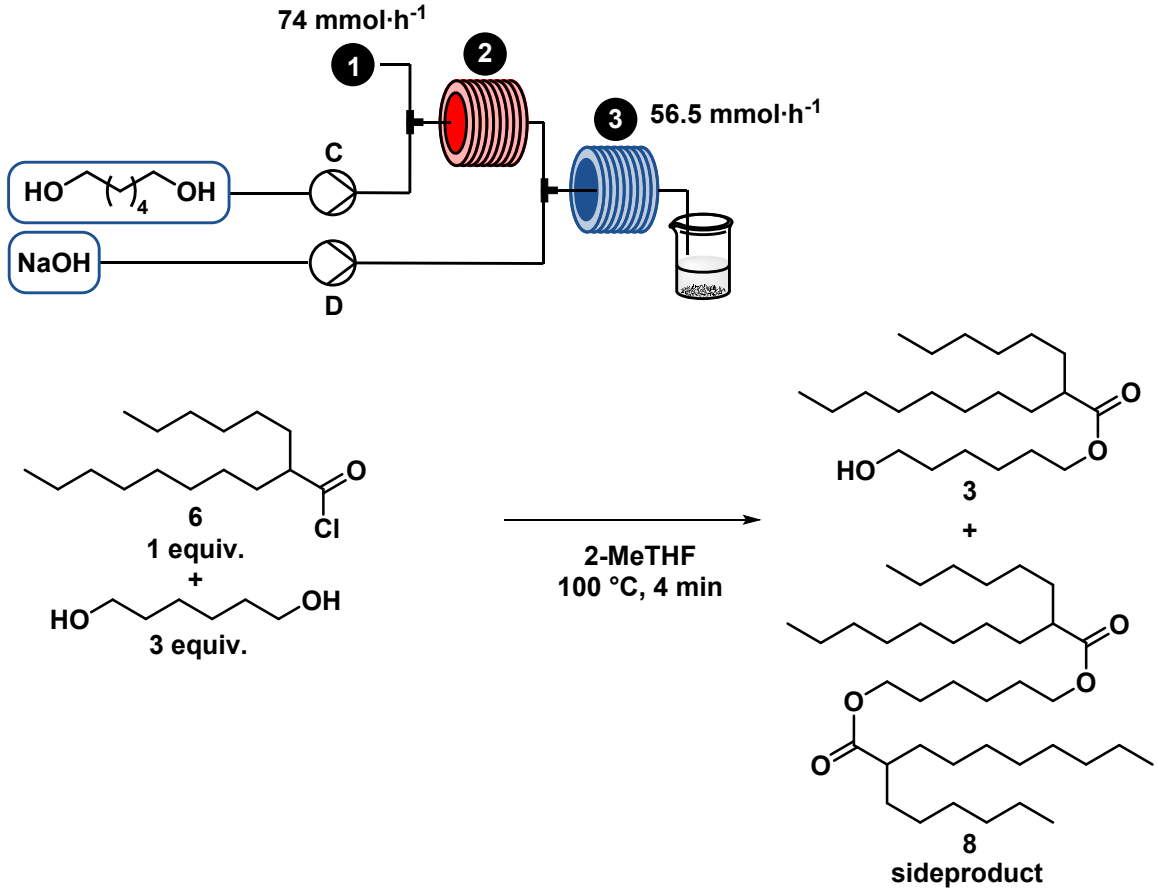


Figure 3: Acid chloride (grey) and carboxylic acid (orange) in IR spectrum (solvent background removed). The split peak of carboxylic acid carbonyl stretch is due to formation of carboxylic acid dimers in solution.

6-hydroxyhexyl-2-hexyldecanoate (**3**)



A solution of hexan-1,6-diol (3 equiv., 48.71 g, 412.2 mmol) in 2-methyltetrahydrofuran (2.50 M) was pumped through 1/16" ETFE tubing (I.D. 1 mm) using an HPLC pump at a flow rate of 1.5 mL·min^{-1} [Line C]. Line C was combined with the output from Reactor 1 via a T-mixer providing a continuous flow of a solution, which was heated at 100 °C in a 10 mL reactor (Reactor 2, 4 min residence time, PTFE tubing). A solution of sodium hydroxide in water (1 M) was pumped through 1/16" ETFE tubing (I.D. 1 mm) using an HPLC pump at a flow rate of 5.5 mL·min^{-1} [Line D]. Line D was combined with the output from Reactor 2 via a T-mixer providing a continuous flow of a solution, which was cooled at 0 °C in a 10 mL reactor in an ice bath (Reactor 3, 1.25 min residence time, ETFE tubing).
The entire system was held under constant pressure using a backpressure regulator (2.8 bar, IDEX 40 PSI, P-761).

*Table 1: Optimization table of hexan-1,6-diol equivalents with **A**) N-Methylimidazole as a base and acylation promoter **B**) without base in the coupling step. Calculations are based on raw areas from LC-ELSD performed on crude reaction mixture, after leaving the reactor (10 min residence time, 100 °C), by 100*monoester(**4**)/(monoester(**4**)+diester(**8**)). Entries with n.d. were not experimentally performed.*

Equiv. hexanediol	**A** [%]	**B** [%]
1.0	25	36
1.2	29	n.d.

1.4	33	n.d.
1.5	n.d.	51
1.6	35	n.d.
1.8	38	n.d.
2.0	43	61
2.4	62	n.d.
2.5	n.d.	73
2.8	53	n.d.
3.0	n.d.	88
3.1	58	n.d.

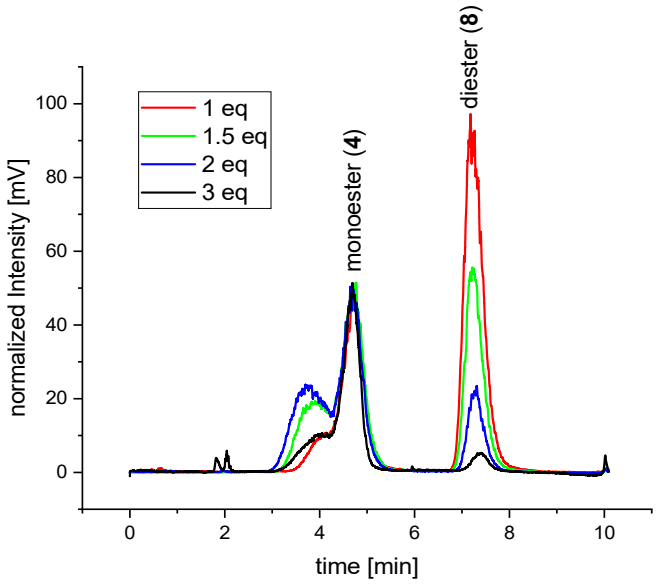


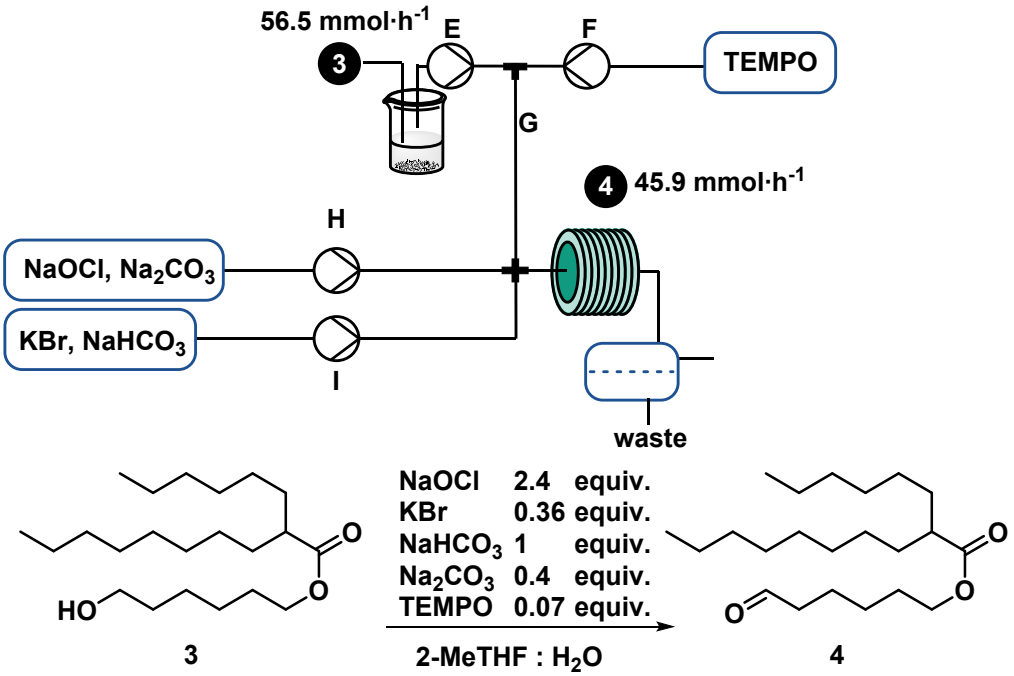
Figure 4: LC-ELSD traces of the crude esterification mixture leaving the neutralization reactor [Stream E].

After a steady state was reached (two residence times, 50.5 min, the combined time of chlorination, esterification and quenching), the biphasic outlet was collected for 30 min and phases separated. The organic phase was used in the next step without any further purification. For analytical purposes, an aliquot (5 mL) of the organic phase (47 mL) was analysed by LC-ELSD and NMR and purified by column chromatography. HPLC analysis of the crude mixture showed a **3**/hexane-1,6-diyl bis(2-hexyldecanoate) ratio of 8:1 (**Figure 4**). As a representative example, after solvent removal under reduced pressure, from 1.603 g of crude material 1.05 g (74%) of **3** were obtained after precipitation of hexane-1,6-diol in hexanes and flash chromatography (hexanes:ethylacetate 0-15%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.07 (t, *J* = 6.6 Hz, 2H), 3.64 (t, *J* = 6.6 Hz, 2H), 2.30 (tt, *J* = 9.1, 5.3 Hz, 1H), 1.60 (m, 6H), 1.48 (s, 1H), 1.44 – 1.32 (m, 6H), 1.25 (d, *J* = 4.5 Hz, 22H), 0.97 – 0.78 (m, 6H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 176.9, 64.1, 63.0, 46.0, 32.8, 32.7, 32.0, 31.8, 29.7, 29.6, 29.39, 29.36, 28.8, 27.62, 27.57, 25.9, 25.5, 22.8, 22.7, 14.3, 14.2.

6-oxohexyl 2-hexyldecanoate (**4**)



The organic phase obtained from the previous step after gravitational separation, was pumped through 1/16" ETFE tubing (I.D. 1 mm) using an HPLC pump at a flow rate of 1.6 mL·min-1 [Line E]. A solution of TEMPO ((2,2,6,6-Tetramethylpiperidin-1-yl)oxyl) in 2-methyltetrahydrofuran (0.50 M) was pumped through 1/16" ETFE tubing (I.D. 1 mm) using an HPLC pump at a flow rate of 0.16 mL·min^{-1} [Line F]. Line F was combined with Line E via a T-mixer [Line G] providing a continuous flow of a solution. A solution of both NaOCl and Na_2CO_3 in water (2 M and 0.3 M respectively) was pumped through 1/16" ETFE tubing (I.D. 1 mm) using a continuous syringe pump at a flow rate of 1.33 mL·min^{-1} [Line H]. A solution of KBr and $NaHCO_3$ in water (0.25 M and 0.7 M respectively) was pumped through 1/16" ETFE tubing (I.D. 1 mm) using an HPLC pump at a flow rate of 1.6 mL·min^{-1} [Line I]. Line G and Line H were combined in a crossmixer with Line I to provide a continuous biphasic stream which was cooled to room temperature by immersion in a water bath in a 11 mL reactor (Reactor 4, 1/16" ETFE tubing (I.D. 1 mm), 2.3 min residence time). The reaction mixture color turned from red (near the cross mixer) to almost colorless (toward the end) through the reactor.

After reaching steady state (two residence times) the biphasic outlet was collected for 10 min. After manual separation of phases, an aliquot of the organic phase (100 µL) was analyzed by quantitative ^{1}H-NMR with 1,3,5-Trimethoxybenzene as internal standard (relaxation delay 10 s, integration of aldehyde proton of **4** @ 9.74 ppm and aromatic protons of internal standard at 6.05 ppm). Collection over 10 minutes gave 13.8 mL of organic phase, with 0.55 M (avg. of three experiments, see Table 2) concentration in **4**, corresponding to 64% overall yield over three steps. The outlet could be separated by membrane separation (SEP-10, Zaiput, Waltham, MA, USA with a Whatman 0.5 µm PTFE membrane cut to size).

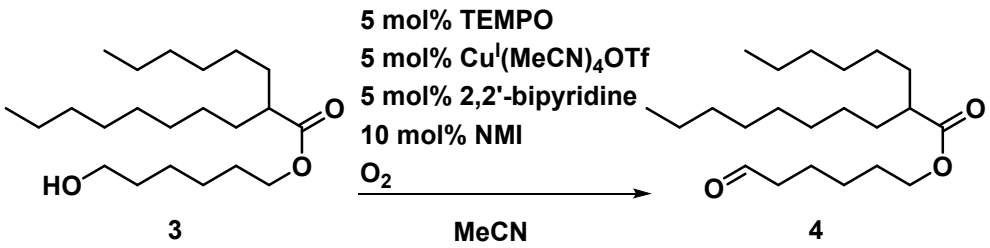
Table 2: Determination of aldehyde concentration in oxidation outlet stream with trimethoxybenzene as internal standard IS. Mean aldehyde molarity is 0.55 M and standard deviation 0.03 M.

Sample	I(**4**) [C**H**O]	I(IS) [C=C-H]	m(trimethoxybenzene) [mg]	c(**4**) [M]
1	1	6.95	21.1	0.54
2	1	6.37	21.1	0.59
3	1	6.73	19.5	0.52

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.75 (t, *J* = 1.7 Hz, 1H), 4.05 (t, *J* = 6.5 Hz, 2H), 2.43 (td, *J* = 7.3, 1.7 Hz, 2H), 2.29 (tt, *J* = 9.0, 5.3 Hz, 1H), 1.70 – 1.50 (m, 6H), 1.46 – 1.33 (m, 4H), 1.31 – 1.16 (m, 20H), 0.85 (t, *J* = 6.8 Hz, 6H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 202.4, 176.8, 63.8, 45.9, 43.8, 32.6, 32.0, 31.8, 29.7, 29.6, 29.4, 29.3, 28.6, 27.6, 27.5, 25.7, 22.8, 22.7, 21.8, 14.22, 14.18.

Alternative preparation of 6-oxohexyl 2-hexyldecanoate (**4**)



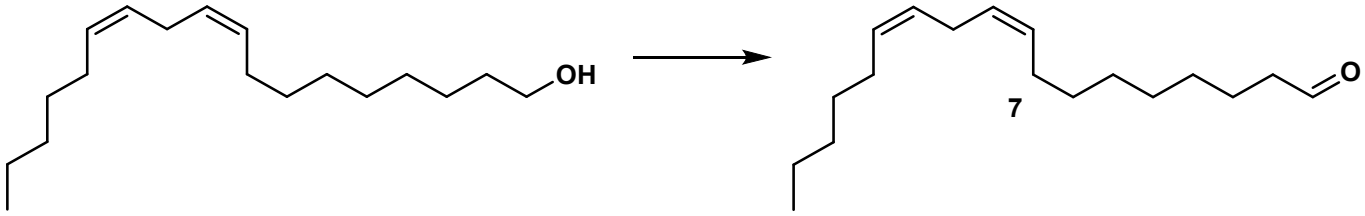
Monoester **3** (1 equiv., 500 mg, 1.4 mmol), 1-Methylimidazole (NMI) (11.2 µL, 0.14 mmol, 10 mol%), TEMPO (10.9 mg, 70 µmol, 5 mol%,) Cu(MeCN)$_4$OTf (26.4 mg, 70 µmol, 5 mol%) and 2,2'Bipyridine (bpy) (10.9 mg, 70 µmol, 5 mol%) were dissolved in 7 mL of degassed MeCN (0.2 M in **3**) under argon. The resulting solution was pumped at variable flow rates by a HPLC pump and joined in a T-mixer with pure oxygen at variable flowrates, controlled by a mass flow controller, and fed into a 10 mL stainless steel reactor (1 mm I.D.) at variable temperature, under a backpressure of 7 bar. The yield of **4** was determined by ^{1}H-NMR analysis of the crude reaction mixture, integrating the aldehyde proton against the overlapping methyl groups at 0.88 ppm (6H).

*Table 3: Optimization table for the Cu^{I}/TEMPO aerobic oxidation of monoester **3** to aldehyde **4**, yield is determined from ^{1}H-NMR after solvent removal by ratio between aldehyde proton and methyl groups of the fatty acid chain at 0.88 ppm in $CDCl_3$.*

Temperature [°C]	additive loading [mol%] NMI/TEMPO/CuOTf/bpy	O_2 [mL/min]	Reagent flowrate [mL/min]	Yield of **4** [%]
60	10/5/5/5	15 - 3.75	0.25	67
60	10/5/5/5	20 -5	1	30
60	10/5/5/5	30 – 7.5	0.5	40
60	10/5/5/5	40 – 10	1	27
60	10/5/5/5	60 - 15	1	40

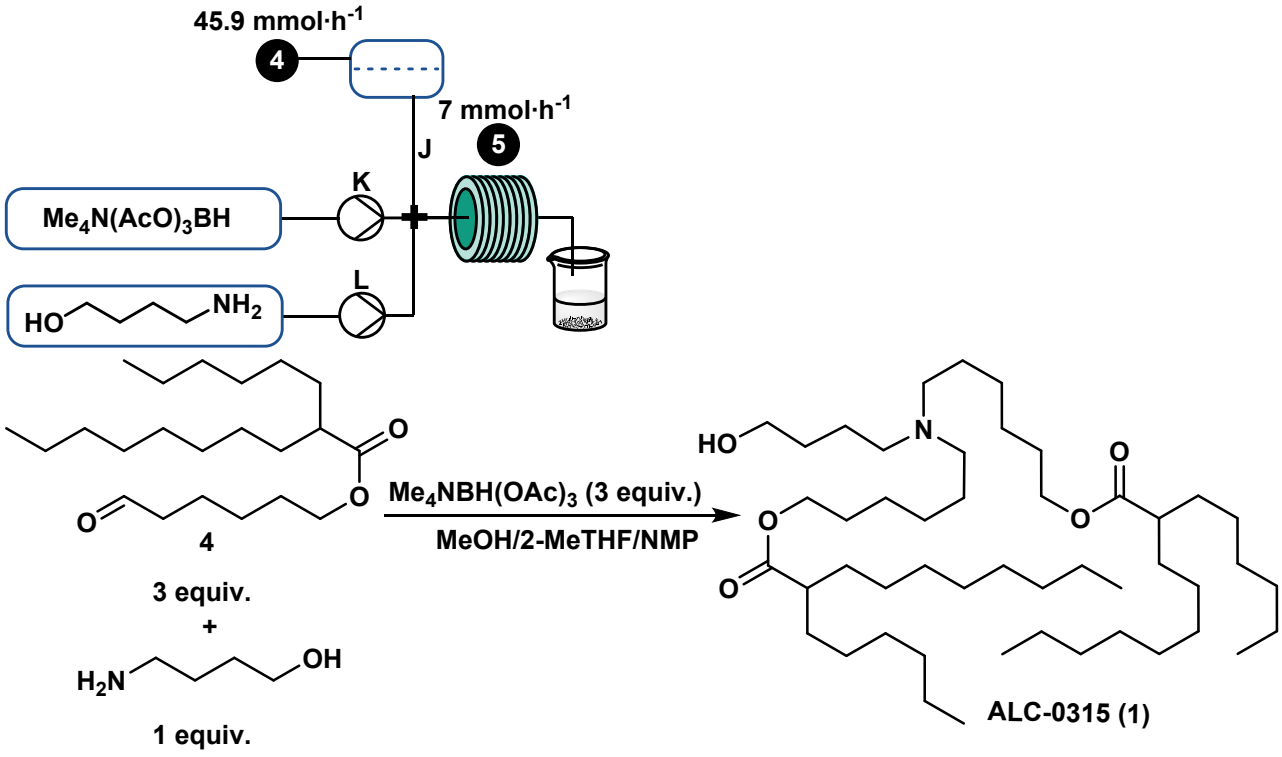
80	10/5/5/5	15	1	25
100	10/5/5/5	15	1	35
60	20/10/10/10	7.5	0.5	63
60	20/10/10/10	3.75	0.25	85
60	20/10/10/10	3	0.2	85

Productivity of aerobic oxidation in flow reported by Fujiwara *et al.*[2]



A productivity of 2 g·h^{-1} of unsaturated aldehyde **7** (MW = 264.45 g·mol^{-1}) in in a 30 m reactor (1 mm ID, 23 mL internal volume) is reported. This corresponds to a productivity of 7.6 mmol·h^{-1} in a 23 mL reactor, or for comparison 3.3 mmol·h^{-1} in a 10 mL reactor.

ALC-0315 ((4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl) bis(2-hexyldecanoate) (**1**)



The organic phase from reactor 4 was dried over Na_2SO_4 and pumped through 1/16" ETFE tubing (I.D. 1 mm) using a syringe pump at a flow rate of 77.7 µL·min^{-1} [Line J]. Tetramethylammonium triacetoxyborohydride in 1-methylpyrrolidin-2-one (0.45 M) was pumped through 1/16" ETFE tubing (I.D. 1 mm) using a syringe pump at a flow rate of 98.4 µL·min^{-1} [Line K]. 4-Aminobutan-1-

ol in methanol (0.2 M) was pumped through 1/16" ETFE tubing (I.D. 1 mm) using a syringe pump at a flow rate of 73.8 µL·min^{-1} [Line L]. The lines J, K, L were joined in a crossmixer, which provided a continuous stream that was cooled to RT in a 4 mL reactor in a water bath (Reactor 5, 1/16" ETFE tubing, I.D. 1 mm, 16 min residence time). After reaching steady state (two residence times), the outlet was collected for 50 min and quenched with water. Hexanes was added, the biphasic mixture was basified to pH 9.5 and the phases separated. The organic phase was dried over Na_2SO_4 and yielded 1.265 g of crude red oil after solvent removal.

An aliquot of crude product (106 mg) was purified on a plug of silica, by sequential washes with hexanes (with 0.1 v% of TFA), hexanes/ethyl acetate (4:1, with 0.1 v% of TFA) and pure ethyl acetate (with 0.1 v% of TFA). Washing the combined fractions of ethyl acetate with Na_2CO_3 and removing the solvent under reduced pressure yielded 22 mg of a slightly yellow oil (yield 47% in last step, based on amino butanol, 31% based on aldehyde).

Note on experimental flow setup for reductive amination:
Overall productivity was extrapolated from small-scale productivity in the last step since the required reactor size of 67 mL was not available and would have required excessive amounts of tubing and aldehyde (85 m w 1 mm I.D.).

Note on column purification of final compound:
Loss of water from the target molecule upon solvent removal (50 °C, reduced pressure) after column purification with TFA as modifier was observed, which gave an additional peak in LC-ELSD analysis, which identity was confirmed by MS, it was therefore necessary to neutralize the eluent before solvent removal by washing with saturated sodium carbonate solution.
NMR spectra of the final compound were acquired in d_8-toluene at low concentration, other solvents failed to produce easily interpretable spectra, presumably due to micelle formation.

Table 4: Optimization table of reductive amination to yield tertiary amine: normalization of raw integral of target tertiary amine to sum of secondary and tertiary amine integrals (uncalibrated LC-ELSD areas).

Residence time [min]	Equiv. **2**	Equiv. TMATAB-H	Tertiary amine [%]
4	1	1.2	41
4	2.2	2.2	90
4	2	2.4	92
4	2.7	2.7	96
4	2.5	3	95
4	2.8	2.8	95
4	3	3	99
8	2.7	4	99
16	2.7	2.7	99

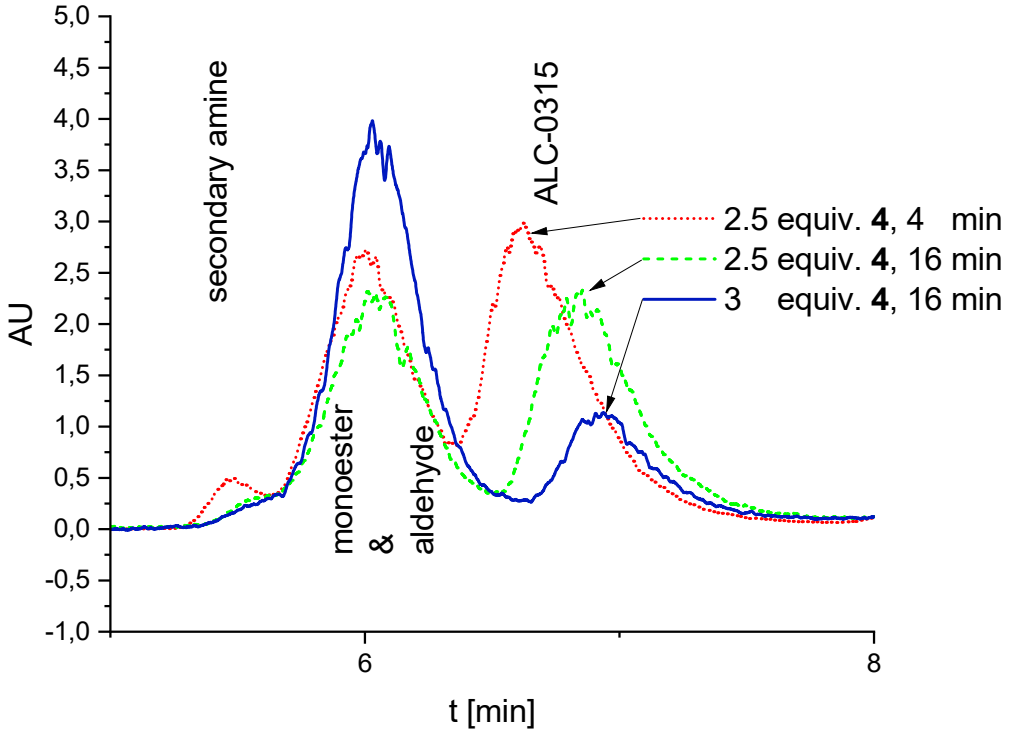


Figure 5: LC-ELSD traces of crude reaction mixture from reductive amination reactor under different synthetic conditions.

^{1}H NMR (400 MHz, Tol-d8) δ = 4.08 (t, *J*=6.7, 4H), 3.57 (t, *J*=5.3, 2H), 2.42 (dp, *J*=9.7, 4.8, 2H), 2.32 – 2.20 (m, 6H), 1.76 (dtt, *J*=13.4, 8.8, 4.5, 4H), 1.60 – 1.12 (m, 64H), 0.95 – 0.89 (m, 12H).

^{13}C NMR (101 MHz, Tol-d8) δ 176.1, 64.4, 63.2, 55.4, 54.6, 46.6, 33.6, 33.4, 32.8, 32.7, 30.6, 30.5, 30.28, 30.25, 29.7, 28.52, 28.46, 28.0, 27.2, 26.84, 26.76, 23.6, 23.5, 14.81, 14.77.

HRMS calculated for $C_{48}H_{95}NO_5$ $[M+H]^+$ *m/z* 766.7284, found 766.7308.

Reductive amination to afford **1** in batch (material for parallel purification optimization)
Aldehyde **4** in 2-MeTHF (30 mL, 0.36 M by qNMR, 10.8 mmol, 3 equiv.) from oxidation outlet was combined with 4-aminobutanol (321 mg, 332 µL, 3.6 mmol, 1 equiv.) in MeOH (15 mL, 0.24 M). Under stirring, STAB-H (2.289 g, 10.8 mmol, 3 equiv.) was slowly added as a solid. A modest exotherm was observed (the flask became warm). After 20 min, HPLC analysis was used to ensure absence of secondary amine. The solution was stored at -20 °C and used for chromatography development and testing.

Purity determination of ALC-0315 by qNMR

A sample of ALC-0315 obtained from Reductive amination to afford **1** in batch (13.1 mg, 17.1 µmol) purified by developed batch chromatography (Purification by Chromatography) was dissolved in toluene-d_8 and CH_2Br_2 (12.1 mg, 69.6 µmol, 4.1 theor. equiv.) added as internal standard. The sample was analyzed by ^{1}H-NMR (interscan delay 16 s, 16 scans) and purity determined ≥ 98 % from the ratio between the target molecule triplet at 3.60 ppm (2H, area = 0.48) and the singlet of internal standard (2H, area = 2.00).

Yield of patent procedure

For comparison of overall yield, the patent procedure[3] performs the reductive amination on a scale of 6.8 mmol of aldehyde (2.4 g). This method yields 0.4 g of ALC-0315 (0.52 mmol). Since two equiv. of aldehyde are required to form one equiv. ALC-0315, the yield in the patent procedure is 15.4% in the last step.

Table 5: Overall yields of literature and patent procedures.

Yield [%]	Remarks	Reference
66		[4]
47		[5]
48		[6]
32	No yield for esterification provided. Overall yield will be lower.	[7]
7-11		[3, 8]

Purification by Chromatography

A Dionex Ultimate® 3000 HPLC system, consisting of a PDA detector (selected λ = 220 nm), a column oven (T = 25 °C), an auto sampler, and a four-channel gradient pump was used for analytical and preparative separations.

Normal-phase conditions for preparative chromatography

Separation was carried out on a normal-phase column (Eurospher-II, 100 Å, 20-45 µm, silica, 100X4 mm) with a mobile phase composed of n-hexane/IPA/EtOH/MeOH and an injection volume of 75 µL. A switching valve (Knauer 6-port 2-position valve) was attached at the outlet of the column for the fractionation of the target compound.

Reversed phase HPLC conditions for analysis

Isocratic elution (water with 0.1 vol.% of trimethylamine / EtOH = 5/95, v/v) was performed at a flowrate of 1.0 mL/min on a reversed-phase column (ReproSil, 100 Å, 5 µm, C18, 100X4 mm) and an injection volume of 0.5 µL.

For the identification of the target and possible impurities, seven standard samples comprising starting material, intermediates and expected impurities (**Figure 6**) were used in the reversed-phase HPLC conditions (**Figure 7**).

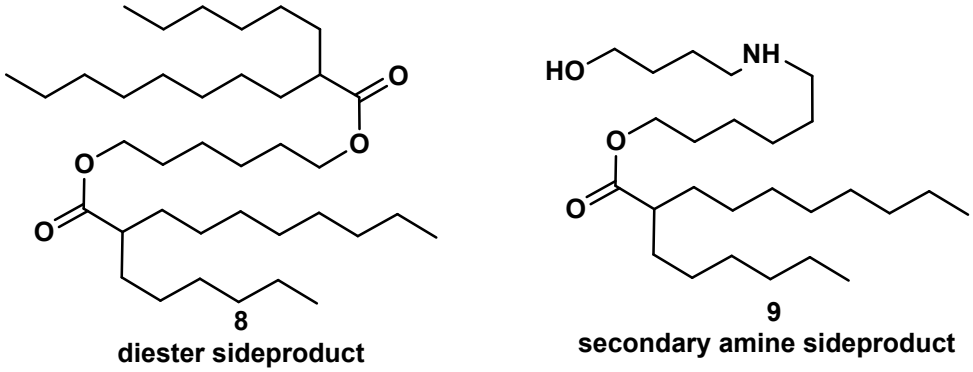


Figure 6 Expected impurities from acylation and reductive amination.

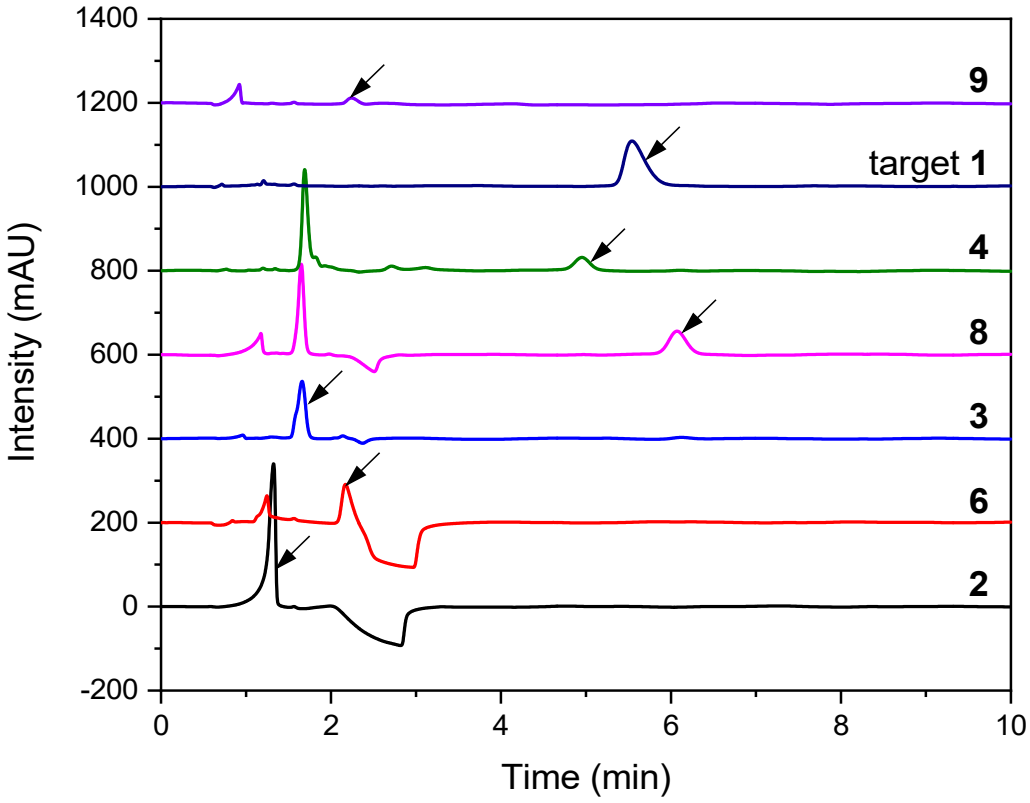


*Figure 7 Reference retention times (marked with black arrow) of starting material **2**, side products, intermediates and product **1**. Injected volume 0.5 µL. Method used according to Reversed phase HPLC conditions for analysis. From bottom to top: hexyldecanoic acid (**2**), acid chloride (**6**), monoester (**3**), diester (**8**), aldehyde (**4**), ALC-0315 (**1**), secondary amine (**9**).*

Workup of crude reaction mixture

The crude reaction mixture containing MeOH and 2-MeTHF was not suitable for normal-phase chromatography. To exchange the solvents to a suitable one (n-Hex) and remove volatile impurities, the following procedure was applied:

Crude reaction mixture (46.61 g, ~100 mL) was evaporated (40 °C, 16 mbar) for 30 min. The resulting residue (17.68 g) was dissolved in n-hexane (40 mL) yielding a cloudy suspension. After precipitation and settling over night at RT, the clear supernatant was decanted from the white crystalline precipitate yielding a yellow, clear solution (**Figure 8**, 44.21 g).

Figure 8 Reaction mixture from batch reductive amination after evaporation and re-dissolving in n-Hex.

For the screening of suitable mobile phase compositions, the mobile phase compositions were linearly changed using four organic solvents, n-Hex, *i*PrOH, EtOH, and MeOH. Since only secondary amine (**9**, **Figure 9**) is more retained than **1** and **1** has a good resolution with other less retained impurities (**2**, **3**, **4**, **6**, **8**), a simple step gradient can achieve complete isolation of the target **1**. The most retained component, secondary amine (**9**) can be eluted in high content of EtOH, so that binary n-Hex/EtOH gradient (Table 5) is sufficient as a mobile phase.

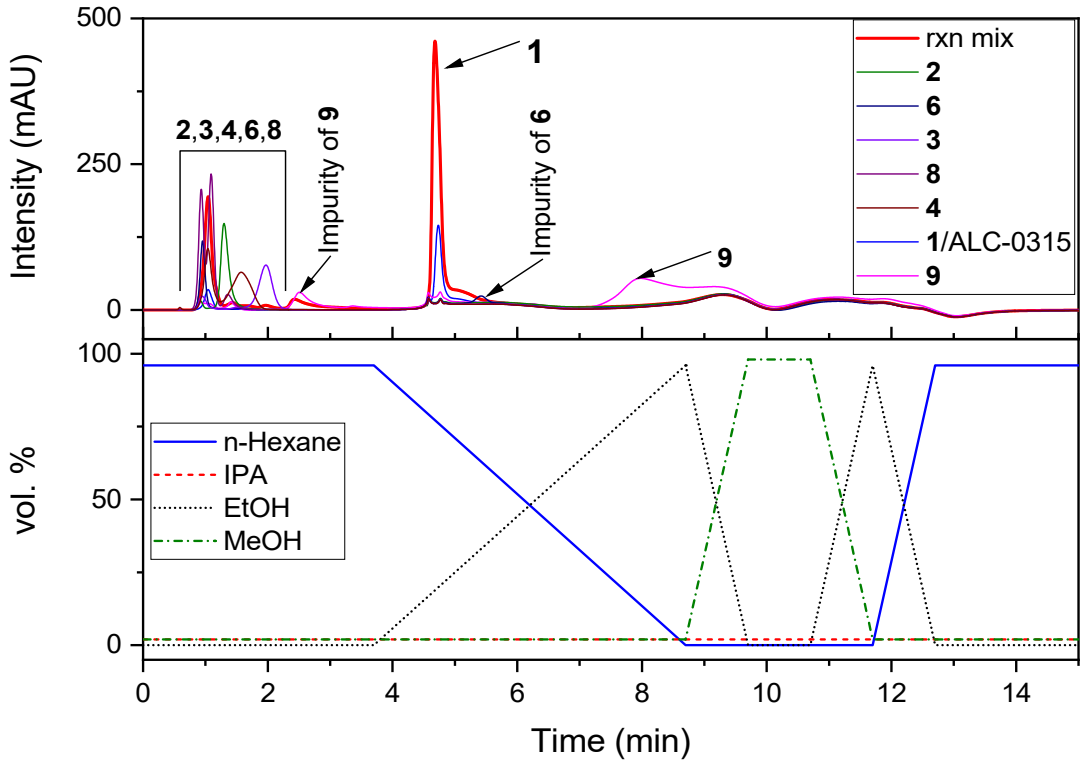


*Figure 9 Chromatogram comparisons of starting materials, intermediates, side products and product with crude reaction mixture under linear gradient conditions. From top to bottom: reaction mixture after solvent exchange (rxn mix), hexyldecanoic acid (**2**), acid chloride (**6**), monoester (**3**), diester (**8**), aldehyde (**4**), ALC-0315 (**1**), secondary amine (**9**).*

Table 6 The gradient elution for the separation of the target compound.

Step	Duration [min]	Mobile phase n-Hex/EtOH (v/v)
Separation	6	97/3
Washing	4	5/95
Equilibration	7	97/3

For the optimal loading of the mixture, the injection volume was gradually increased up to 100 µL (**Figure 10**), and the injection volume was fixed to 75 µL for a train of 50 runs (**Figure 11**). The target fractions were collected and solvent removed under reduced pressure (40 °C, 25 mbar). The purity of the target compound was assessed by ^{1}H-NMR (> 98 %, see Purity determination of ALC-0315 by qNMR).

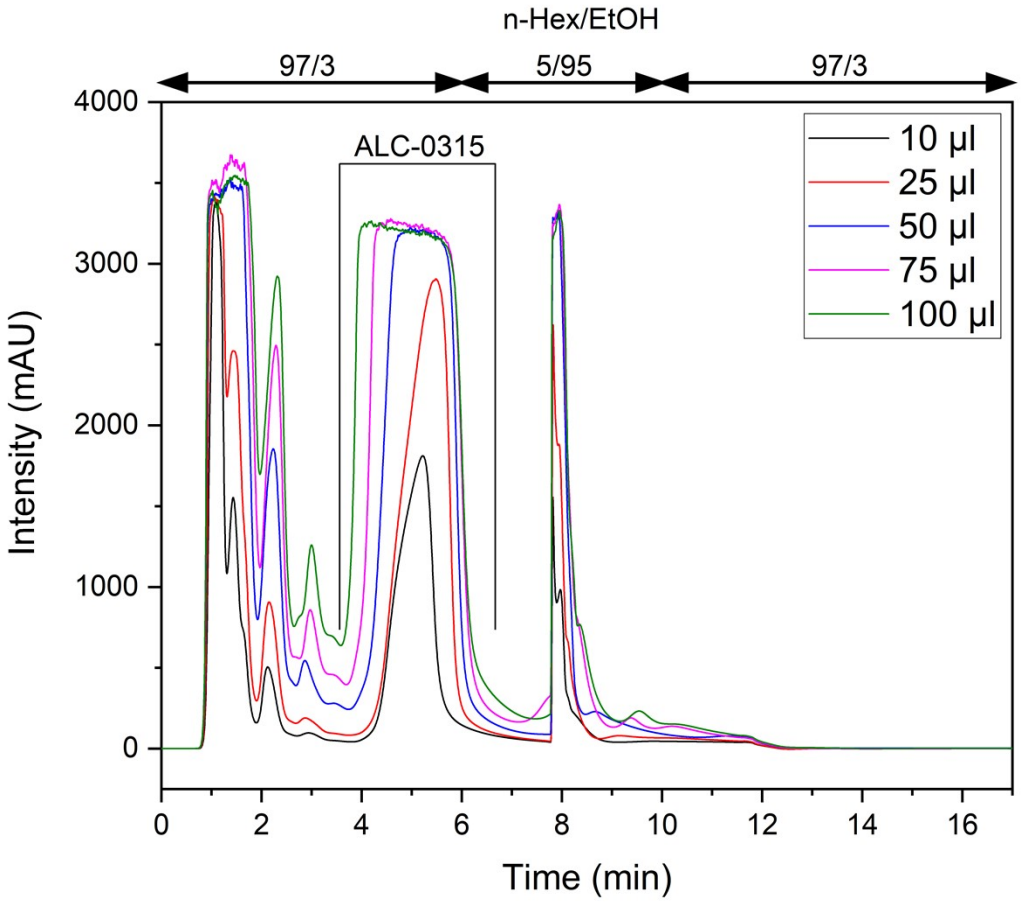


Figure 10 Chromatogram comparisons of the volume overloading test. Injection of different amounts of reaction mixture in order to determine ideal loading volume. Ideal loading volume is determined as 75 µL from this experiment.

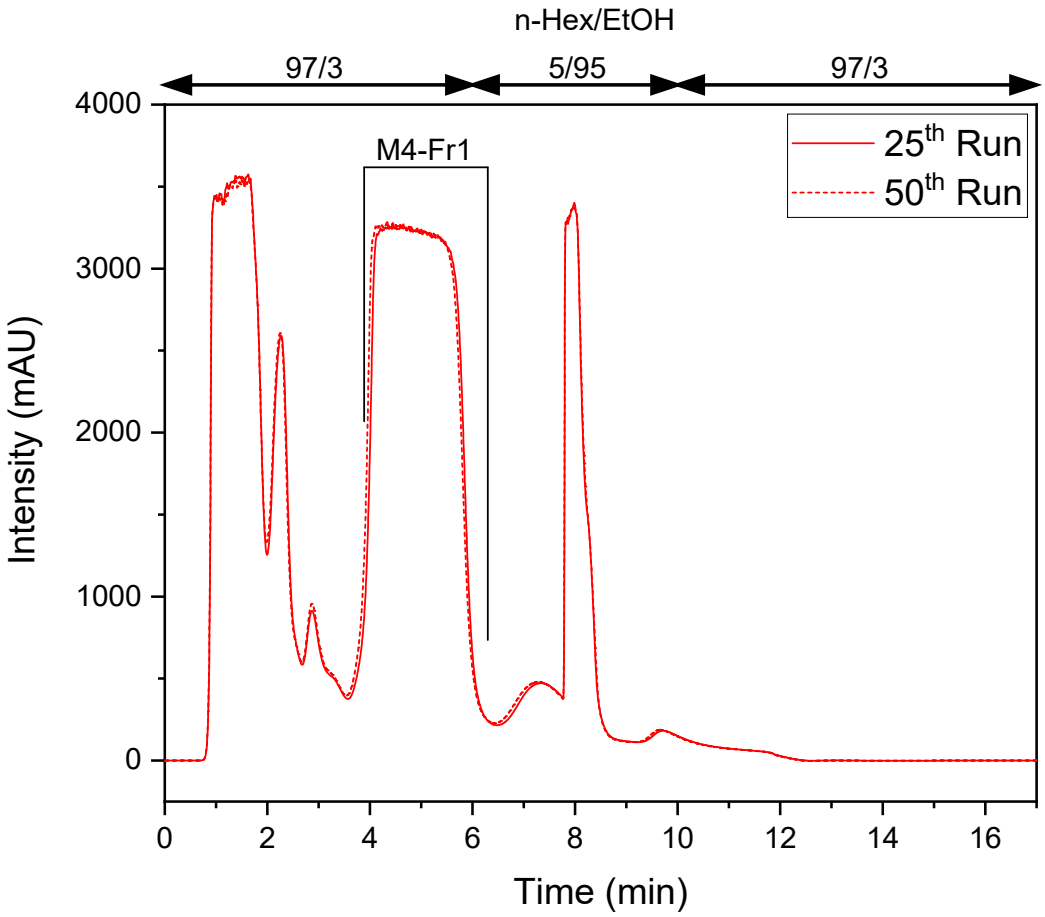
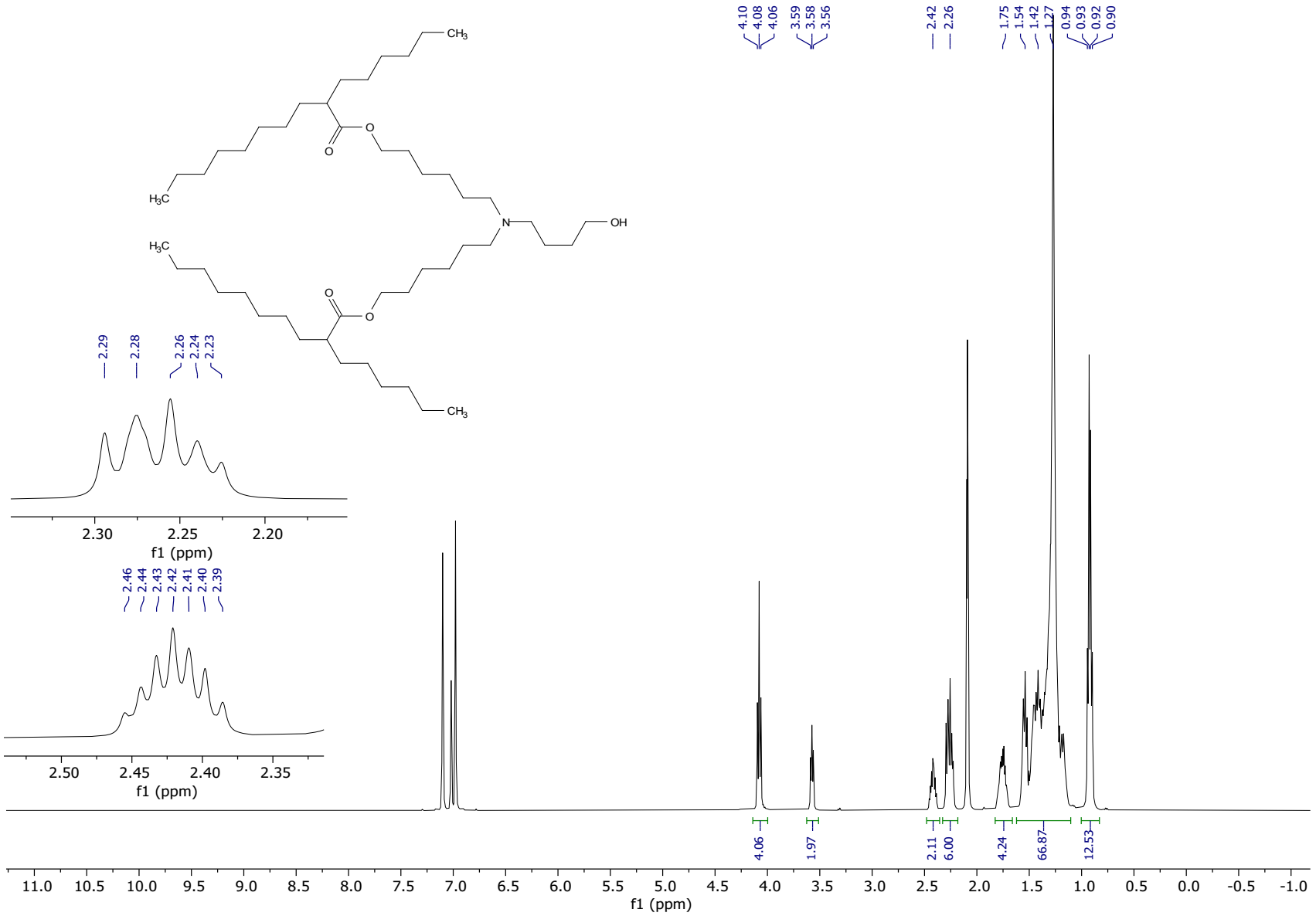
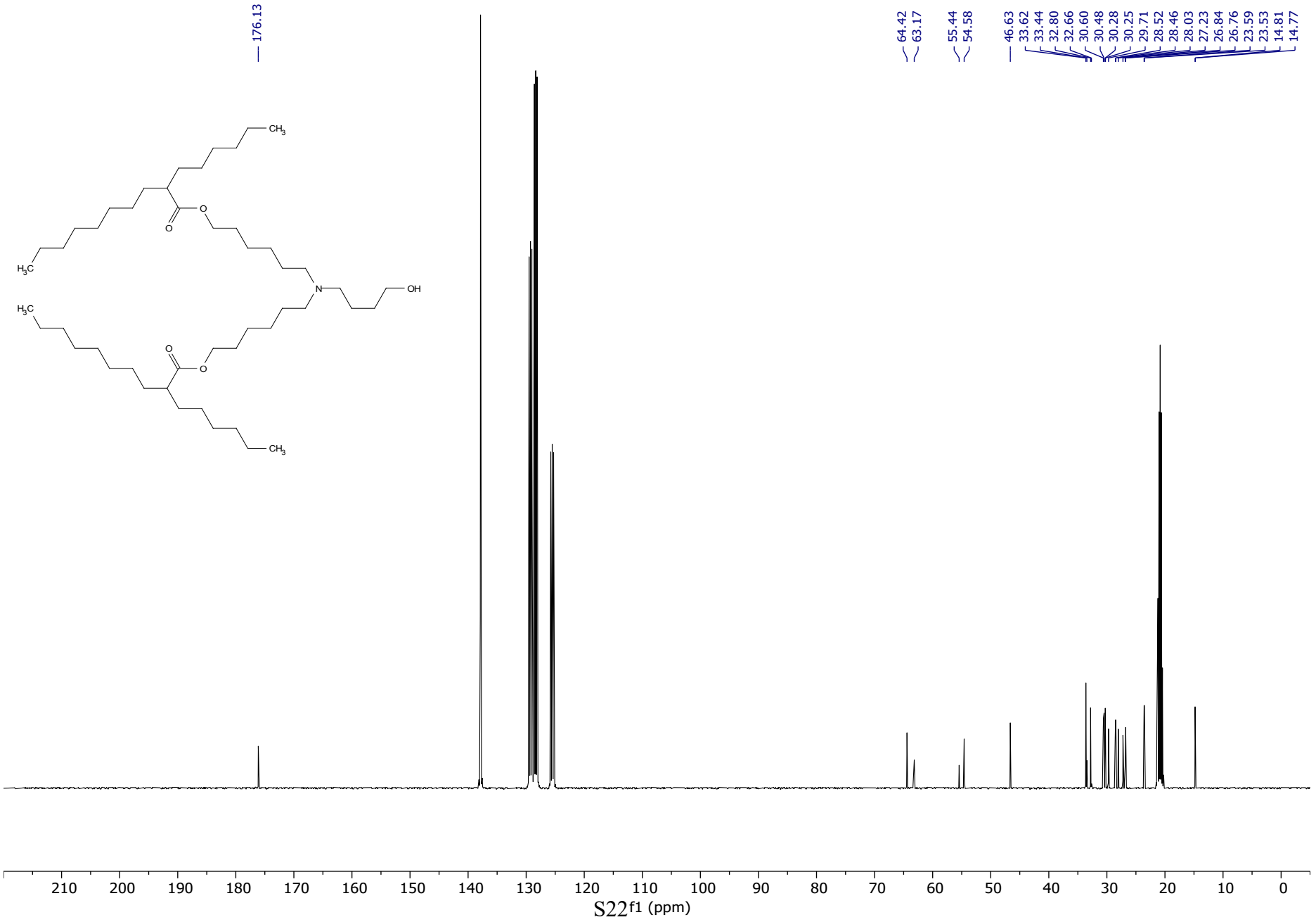


Figure 11: Reproducibility studies of applied gradient and injection volume show good reproducibility of elution profile over 50 injections.

Spectra

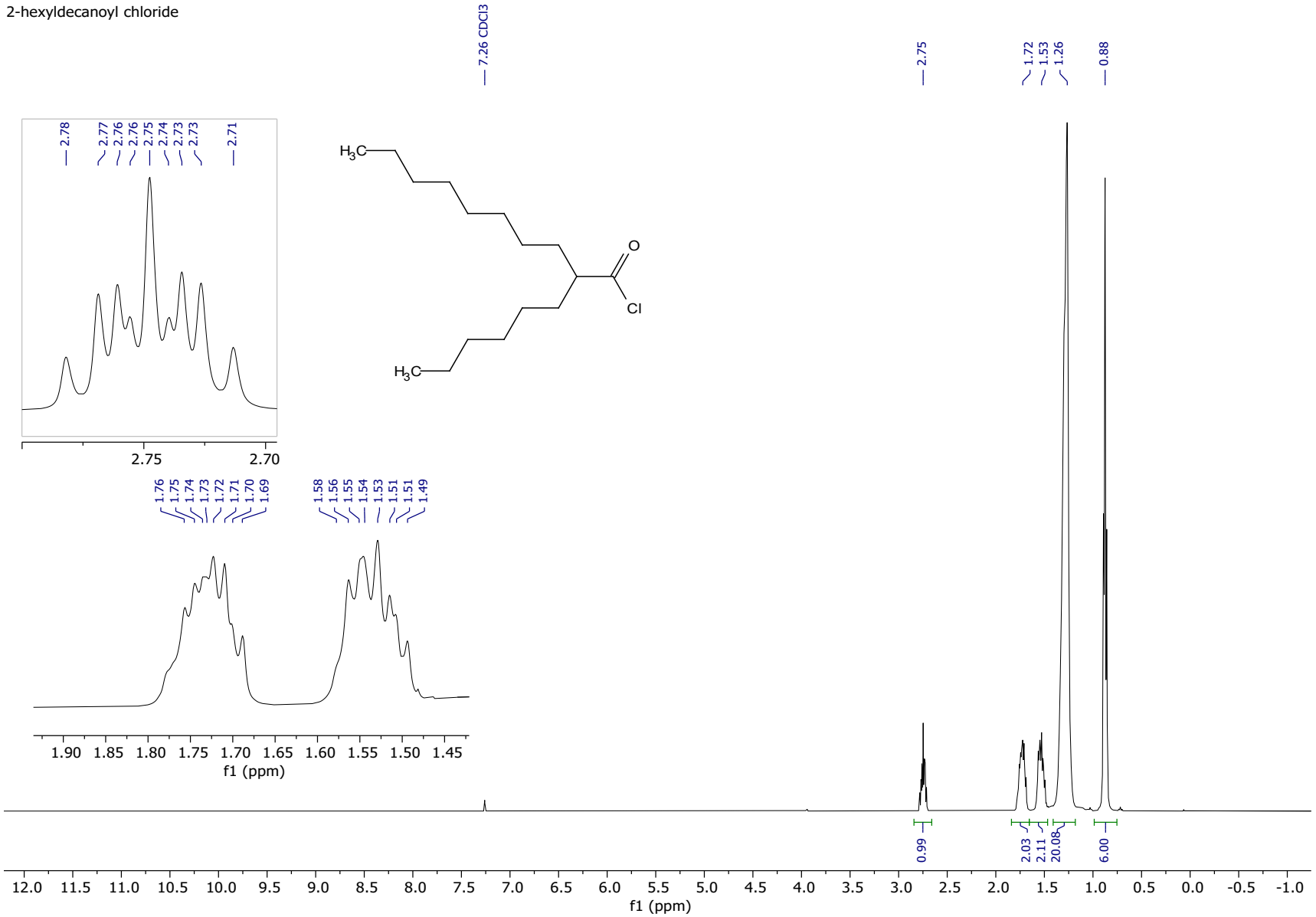
^{1}H (400 MHz, 298 K) and ^{13}C (100.6 MHz, 298 K) NMR spectra of ALC-0315 (**1**) in d_8-toluene



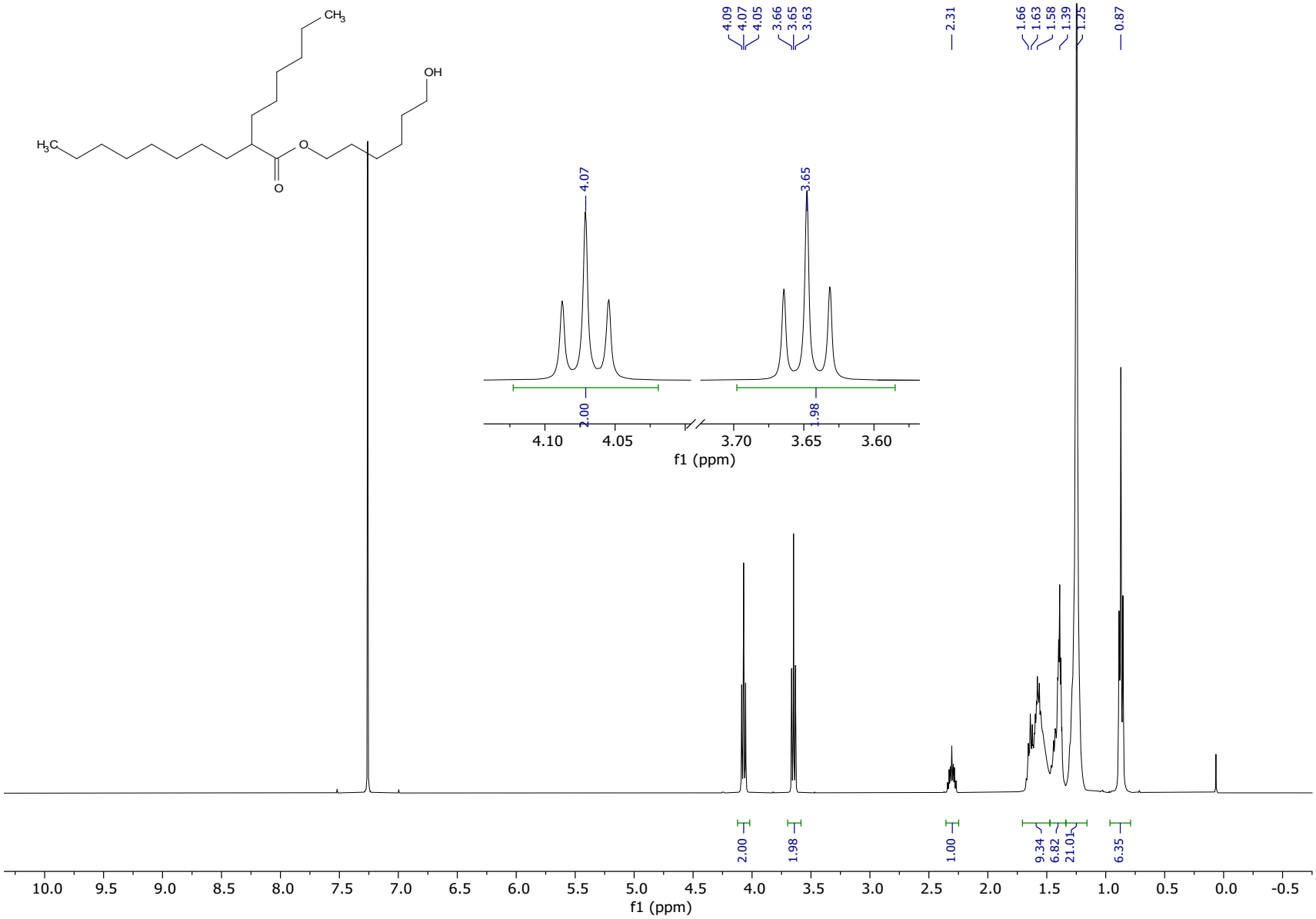

176.13
64.42
63.17
55.44
54.58
46.63
33.62
33.44
32.80
32.66
30.60
30.48
30.28
30.25
29.71
28.52
28.46
28.03
27.23
26.84
26.76
23.59
23.53
14.81
14.77
CH3
O
O
H3C
N
OH
H3C
O
O
CH3
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
S22f1 (ppm)

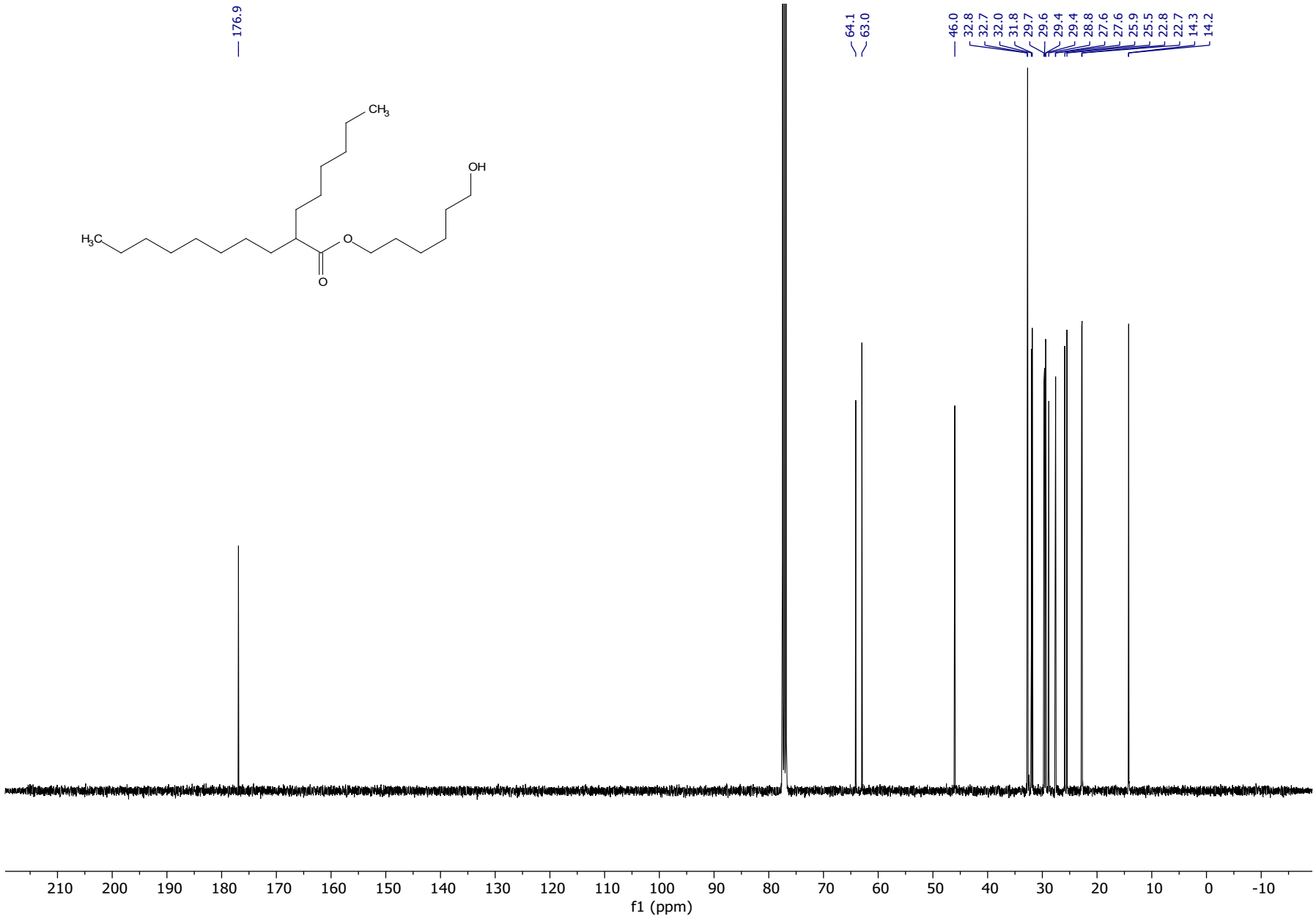
^{1}H NMR spectrum (400 MHz, 298 K) of **6** in $CDCl_3$

2-hexyldecanoyl chloride

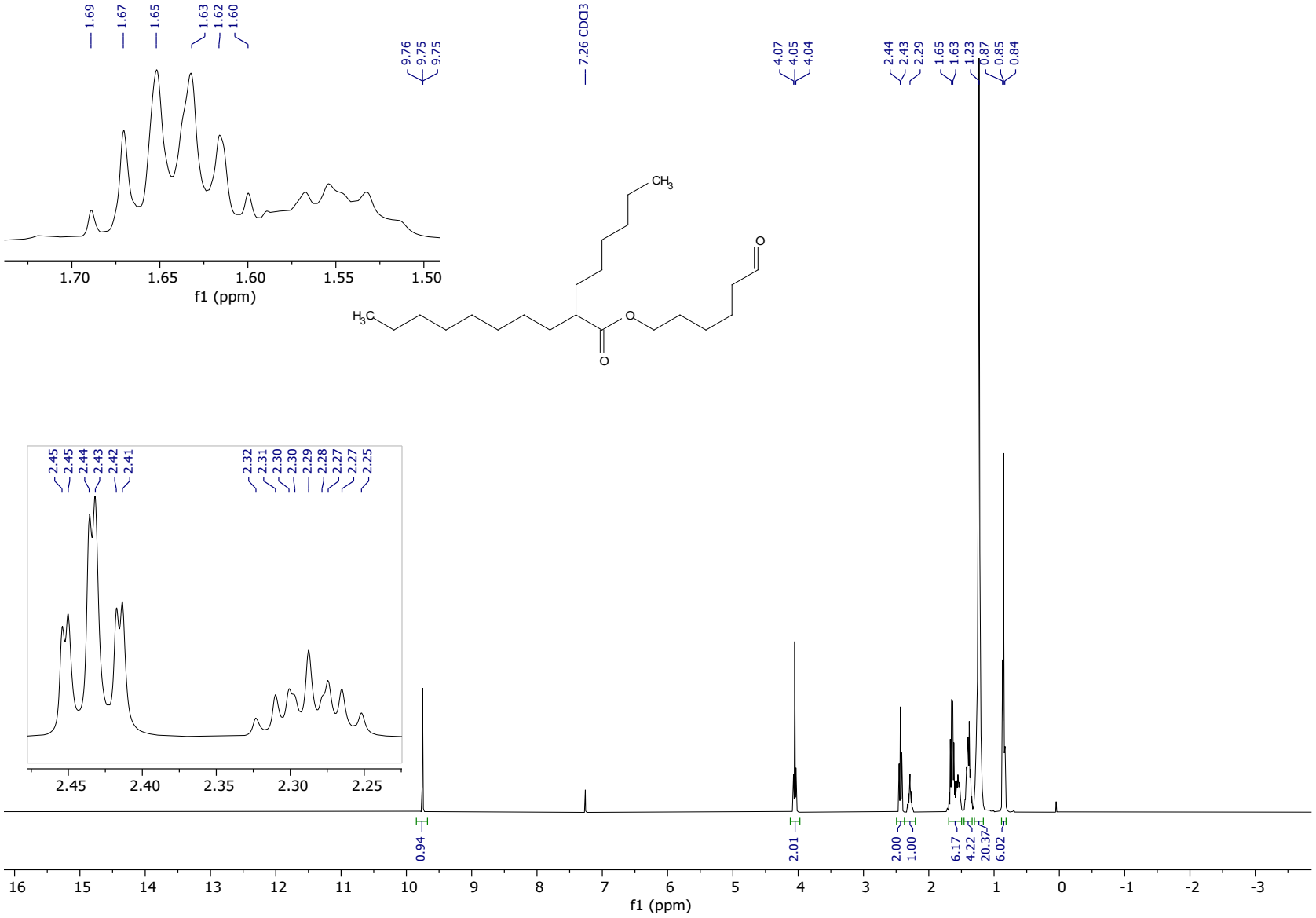


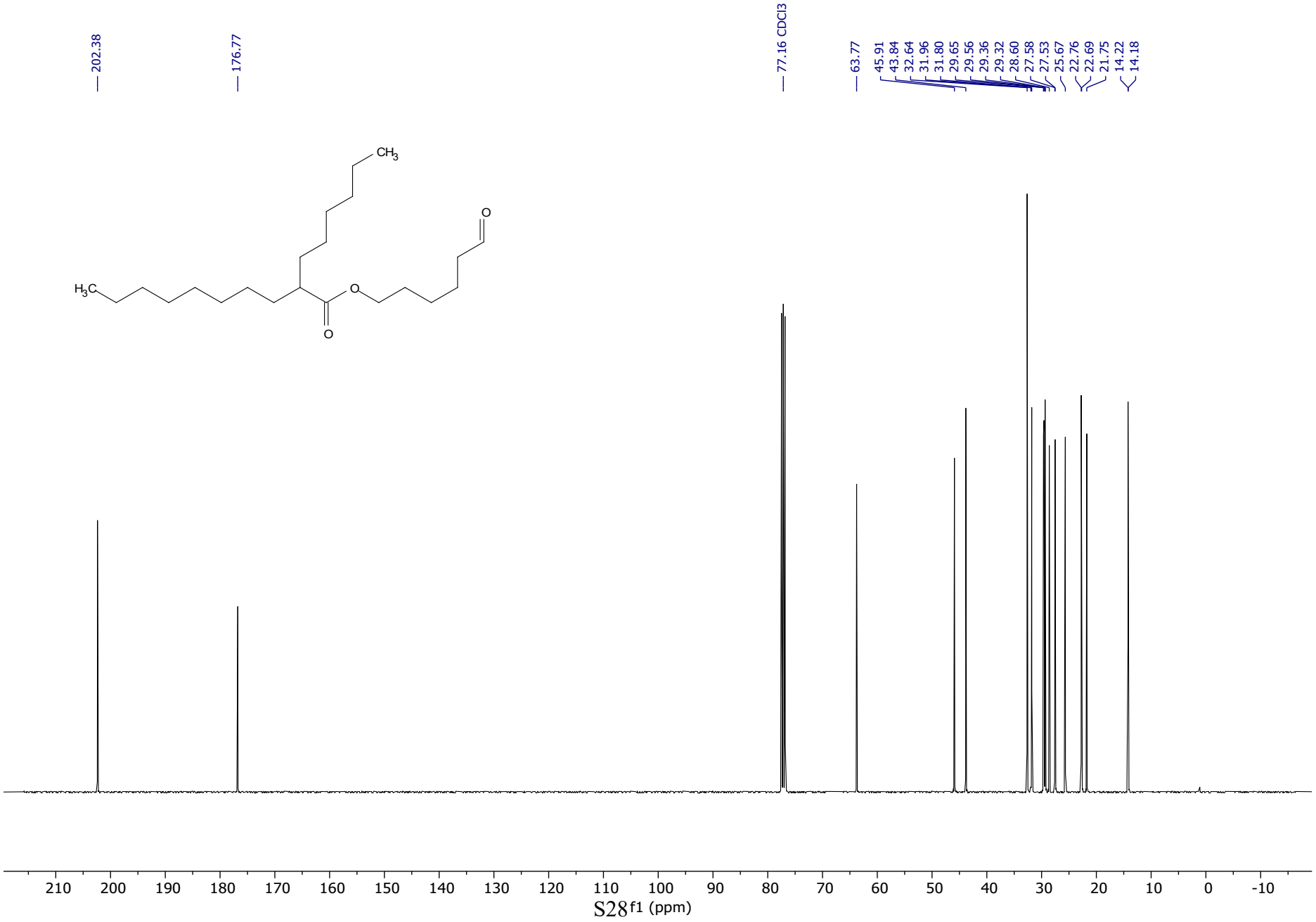
^{1}H (400 MHz, 298 K) and ^{13}C (100.6 MHz, 298 K) NMR spectra of **3** in $CDCl_3$




176.9
64.1
63.0
46.0
32.8
32.7
32.0
31.8
29.7
29.6
29.4
29.4
28.8
27.6
27.6
25.9
25.5
22.8
22.7
14.3
14.2
CH3
OH
H3C
O
O
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

^{1}H (400 MHz, 298 K) and ^{13}C (100.6 MHz, 298 K) NMR spectra of **4** in $CDCl_3$




202.38
176.77
77.16 CDCl3
63.77
45.91
43.84
32.64
31.96
31.80
29.65
29.56
29.36
29.32
28.60
27.58
27.53
25.67
22.76
22.69
21.75
14.22
14.18
CH3
H3C
O
O
O
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

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