Supplementary Information

One-pot biocatalytic amine transaminase/acyl transferase cascade for aqueous formation of amides from aldehydes or ketones

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Experimental

Enzyme cloning, expression and purification

The cloning of the gene of *Silicibacter pomeroyi* amine transaminase (*Sp*-ATA) has been described elsewhere.[1] The codon-optimized gene of *Mycobacterium smegmatis* acyl transferase (*Ms*AcT) was purchased cloned into pET-28a(+) from GenScript (Piscataway, U.S.A.). Expression of *Sp*-ATA was performed by inoculating 20 mL LB-medium (100 µg/mL ampicillin) with *Escherichia coli* (*E. coli*) BL21 (DE3) cells containing the *Sp*-ATA encoding plasmid. The culture was incubated at 37 °C and 210 rpm overnight. 180 mL LB-medium (100 µg/mL ampicillin and 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG)) was then inoculated with the overnight culture and incubated at 25 °C and 140 rpm for 24 h. After expression, the cells were harvested by centrifugation (8000 rpm for 15 min). The cell pellet was dissolved in Immobilized Metal ion Affinity Chromatography (IMAC) binding buffer (sodium phosphate buffer (20 mM, pH 7.4) and NaCl (0.5 M)) and cells were disrupted by sonication. Cell debris was removed by centrifugation (20000 rpm form 15 min) and the supernatant was collected and filtered (0.45 µm). The enzyme was then purified by IMAC according to the manufacturers' protocol. Pyridoxal-5´-phosphate (PLP) was added in an excess (1 mM) and the solution was incubated in room temperature for 30 minutes. Finally, a buffer change to CHES buffer (0.2 M, pH 10) was performed by running the solution through a PD-10 column according to the manufacturers' protocol. The enzyme solution was stored at 4 °C. Expression of *Ms*AcT was performed by inoculating 10 mL LB-medium (50 µg/mL kanamycin) with *E. coli* BL21 (DE3) cells containing the *Ms*AcT encoding plasmid. The culture was incubated at 37 °C and 210 rpm overnight. 1 L LB-medium (50 µg/mL kanamycin) was inoculated with the overnight culture and incubated at 37 °C and 210 rpm until an optical density (OD_{600}) of 0.6-0.8 was reached. 1 mM IPTG was added and the culture was incubated at 30 °C and 140 rpm for 18h. Harvesting, disruption and IMAC purification was performed as with *Sp*-ATA. After purification, the buffer was changed into potassium phosphate buffer (20 mM, pH 7.2) using a PD-10 column. The enzyme solution was stored at -20 °C.

Determination of enzyme activity

The activity of *Sp*-ATA was determined using the acetophenone assay[2] with 2.5 mM (*S*)-1-phenylethylamine and 2.5 mM sodium pyruvate. Production of acetophenone was followed spectrophotometrically at 245 nm using a Varian Cary 50 Bio UV-Visible Spectrophotometer. The reaction was performed in CHES buffer (0.2 M, pH 10). Activity of *Ms*AcT was determined by hydrolysis of methyl acetate (2 mM). The production of acetic acid was followed

spectrophotometrically as the decrease in absorption due to the protonation of the pH-indicator *p*-nitrophenol (0.1 mM) at 410 nm. The reaction was performed in potassium phosphate buffer (5 mM, pH 7.2, 1% AcN)

Product analysis

All product analysis was performed with gas chromatography (GC) using a Hewlett Packard 5890 equipped with an Agilent CP-ChiraSil-DEX CB column (25m x 0.32 mm x 0.25 µm) and a flame ionization detector (FID). Conversion into achiral and enantiomerically pure chiral amides was determined with the following temperature gradient: starting temperature 50 °C, 20 °C/min to 225 °C, 225 °C was kept constant for 5 min. Retention times: decane (4.36 min), *N*-benzylacetamide (8.82 min), *N*-benzyl-2-methoxyacetamide (9.18 min), (*S*)-*N*-(hexan-2-yl)-2-methoxyacetamide (6.97 min) and (*R*)-*N*-(hexan-2-yl)-2-methoxyacetamide (7.06 min). Conversion and enantiomeric excess (*ee*) of mixtures of chiral amides was determined with the following temperature gradient: starting temperature 50 °C, 1 °C/min to 225 °C, 225 °C was kept constant for 5 min. Retention times: decane (18.00 min), (*S*)-*N*-(hexan-2-yl)-2-methoxyacetamide (55.22 min) and (*R*)-*N*-(hexan-2-yl)-2-methoxyacetamide (58.62 min).

***Ms*AcT pH optimum**

The pH optimum of *Ms*AcT was investigated by running a reaction with 20 mM benzylamine, 1% methyl acetate, 1.6 U/mL *Ms*AcT and Davies buffer[3] pH 7-12. The reactions were stopped by acidification with HCl (6 M) after 5 minutes. The product amide was then extracted to chloroform containing 5 mM decane. GC analysis was performed on the chloroform layer to determine conversion to *N*-benzylacetamide.

***Ms*AcT reactions**

The capability of *Ms*AcT to catalyze the amidation of benzylamine was investigated by running a reaction with 20 mM benzylamine, 1% methyl acetate or methyl methoxyacetate, 1.6 U/mL *Ms*AcT and CHES buffer (0.2 M, pH 10). Reactions were followed over time by withdrawing samples for GC analysis. Similarly, the capability of *Ms*AcT to catalyze the amidation of 2-aminohexane was investigated by running a reaction with 20 mM (*S*)-, (*R*)- or (*S/R*)-2-aminohexane or (*S*/*R*)-1-phenylethylamine, 2% methyl methoxyacetate, 1.6 U/mL *Ms*AcT and CHES buffer (0.4 M, pH 10). Reactions were followed over time by withdrawing samples for GC analysis.

Cascade optimization

The enzyme cascade was optimized by running reactions with 20 mM benzaldehyde, 0.5 M L-alanine, 1-2% methyl acetate or methyl methoxyacetate, *Sp*-ATA (0.03, 0.3 and 3 U/mL), *Ms*AcT (0.016, 0.16 and 1.6 U/mL) and CHES buffer (0.2 or 0.4 M, pH 10). Samples were collected for GC analysis after 18h.

Cascade for the synthesis of *N*-phenylethyl-2-methoxyacetamide, *N*-butyl-2-methoxyacetamide and *N*-heptyl-2-methoxyacetamide

The enzyme cascades were performed by running reactions with 20 mM aldehyde (phenylacetaldehyde, butanal or heptanal), 0.5 M L-alanine, 2% v/v methyl methoxyacetate, 3 U/mL *Sp*-ATA, 1.6 U/mL *Ms*AcT and CHES buffer (0.4 M, pH 10). 5% DMSO was added to the reactions with phenylacetaldehyde and heptanal. Samples were collected for GC analysis after 18h.

Cascade for the synthesis of (*S*)-*N*-(hexan-2-yl)-2-methoxyacetamide
The enzyme cascade was performed by running a reaction with 20 mM 2-hexanone, 0.5 M L-alanine, 2% v/v methyl methoxyacetate, 3 U/mL *Sp*-ATA, 1.6 U/mL *Ms*AcT and CHES buffer (0.4 M, pH 10). Samples were collected for GC analysis after 18h.

***Sp*-ATA tolerance against methyl methoxyacetate**
The residual activity of *Sp*-ATA in varying concentrations of methyl methoxyacetate was investigated by performing the acetophenone assay[2] with 2.5 mM (*S*)-1-phenylethylamine, 2.5 mM sodium pyruvate, 0-6% v/v methyl methoxytacetate and CHES buffer (0.2 M, pH 10).

Transamination of benzaldehyde
Transamination of benzaldehyde was performed by running a reaction with 20 mM benzaldehyde, 0.5 M L-alanine, 3 U/mL *Sp*-ATA and CHES buffer (0.4 M, pH 10). The reaction was analyzed by GC after 18h.

Preparative synthesis of *N*-benzyl-2-methoxyacetamide
The preparative synthesis of *N*-benzyl-2-methoxyacetamide was performed by adding 20 mM benzaldehyde (1 mmol), 0.5 M L-alanine (25 mmol), 2% v/v methyl methoxyacetate (10.1 mmol), 3 U/mL *Sp*-ATA and 1.6 U/mL *Ms*AcT to a total volume of 50 mL in CHES buffer (0.4 M, pH 10). The reaction was incubated in room temperature with slow mixing (120 rpm) for 24h. The reaction was stopped by addition of HCl (6M) to a pH of appr. 2 and the product was extracted with chloroform. The chloroform was thereafter mixed with sodium phosphate buffer (500 mM, pH 12). Finally, the chloroform was dried with $MgSO_4$, filtered and evaporated leaving the product amide.

Reference synthesis of *N*-benzylacetamide
Methyl acetate (30 mmol) was mixed with benzylamine (15 mmol) and 50 mg Novozym 435 in a 10 ml round-bottom flask. After 3h at 50 °C with magnetic stirring the enzyme was filtered off and the remaining ester was evaporated in a rotary evaporator. The remaining mixture was further purified by extraction $CHCl_3$:H_2O pH 7. The chloroform was subsequently dried with $MgSO_4$, filtered and evaporated. Finally, the amide was recrystallized from warm hexane.

Reference synthesis of *N*-benzyl-2-methoxyacetamide
Methyl methoxyacetate (16 mmol) was mixed with benzylamine (15 mmol) and 50 mg Novozym 435 in a 10 ml round-bottom flask. The reaction was left open for 3h at 50 °C with magnetic stirring. The enzyme was thereafter filtered off and the remaining ester was evaporated in a rotary evaporator. The amide was further purified by extraction $CHCl_3$:H_2O pH 7. The chloroform was subsequently dried with $MgSO_4$, filtered and evaporated leaving the product amide.

Reference synthesis of *N*-phenylethyl-2-methoxyacetamide
Methyl methoxyacetate (16 mmol) was mixed with 2-phenylethylamine (13 mmol) and 50 mg Novozym 435 in a 10 ml round-bottom flask. The reaction was left open for 24h at 40 °C with

magnetic stirring. The enzyme was thereafter filtered off and the reaction mixture was purified by extraction, first with a $CHCl_3$:H_2O (100 mM HCl) mixture and thereafter with $CHCl_3$:H_2O (saturated $KHCO_3$). The chloroform was subsequently dried with $MgSO_4$, filtered and evaporated leaving the product amide.

Reference synthesis of *N*-butyl-2-methoxyacetamide

Methyl methoxyacetate (16 mmol) was mixed with butylamine (16 mmol) and 50 mg Novozym 435 in a 10 ml round-bottom flask. The reaction was left open for 24h at 40 °C with magnetic stirring. The enzyme was thereafter filtered off and the reaction mixture was purified by extraction, first with a $CHCl_3$:H_2O (100 mM HCl) mixture and thereafter with $CHCl_3$:H_2O (saturated $KHCO_3$). The chloroform was subsequently dried with $MgSO_4$, filtered and evaporated leaving the product amide.

Reference synthesis of *N*-heptyl-2-methoxyacetamide

Methyl methoxyacetate (16 mmol) was mixed with heptylamine (11 mmol) and 50 mg Novozym 435 in a 10 ml round-bottom flask. The reaction was left open for 24h at 40 °C with magnetic stirring. The enzyme was thereafter filtered off and the reaction mixture was purified by extraction, first with a $CHCl_3$:H_2O (100 mM HCl) mixture and thereafter with $CHCl_3$:H_2O (saturated $KHCO_3$). The chloroform was subsequently dried with $MgSO_4$, filtered and evaporated leaving the product amide.

Reference synthesis of (*S*)- and (*R*)-*N*-(hexan-2-yl)-2-methoxyacetamide

Methyl methoxyacetate (9 mmol) was mixed with (S)- or (R)-2-hexylamine (3 mmol) in a 5 ml round-bottom flask. The reaction was left open for 24h at 50 °C with magnetic stirring either catalyzed by Novozym 435 for the R- or HCl for the S-enantiomer. The reaction mixtures were purified by extraction, first with a $CHCl_3$:H_2O (100 mM HCl) mixture and thereafter with $CHCl_3$:H_2O (saturated $KHCO_3$). The chloroform was subsequently dried with $MgSO_4$, filtered and evaporated leaving the product amide.

NMR analysis

The identities of the synthesized reference compounds and the product of the preparative synthesis were confirmed by ^{1}H-NMR on a Bruker AM-400 or Bruker AM-500 and analyzed with MestReNova 7.1.1. All chemical shifts are given relative to tetramethylsilane.

Results

*Ms*AcT pH-optimum

The pH-optimum of *Ms*AcT was determined by measuring the conversion into *N*-benzylacetamide from benzylamine and methylacetate. The optimal pH for amidation was determined to be 11 (Figure S1).

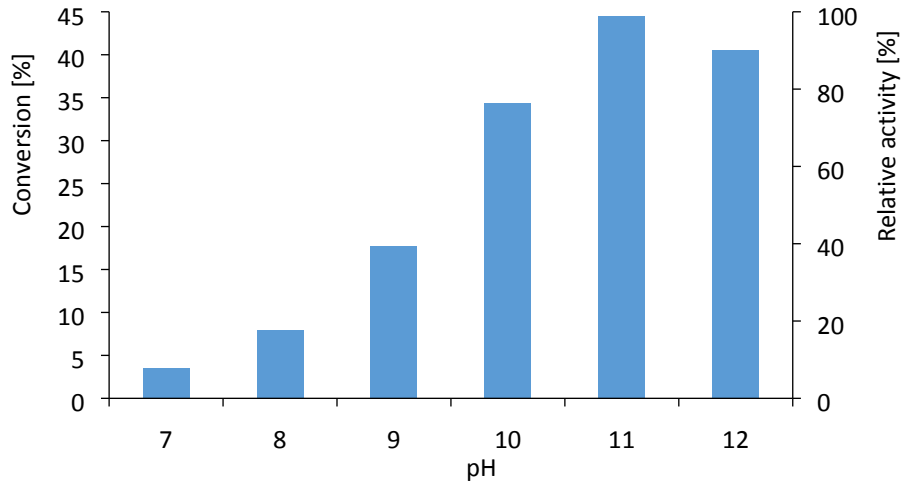


Fig. S1. pH-profile for amidation catalyzed by *Ms*AcT. The reaction between benzylamine (20 mM) and methyl acetate (1% v/v) was stopped after 5 min and conversion into *N*-benzylacetamide was determined.

Sp-ATA tolerance against methyl methoxyacetate

The residual activity of *Sp*-ATA in the presence of different amounts of methyl methoxyacetate was determined (Figure S2).

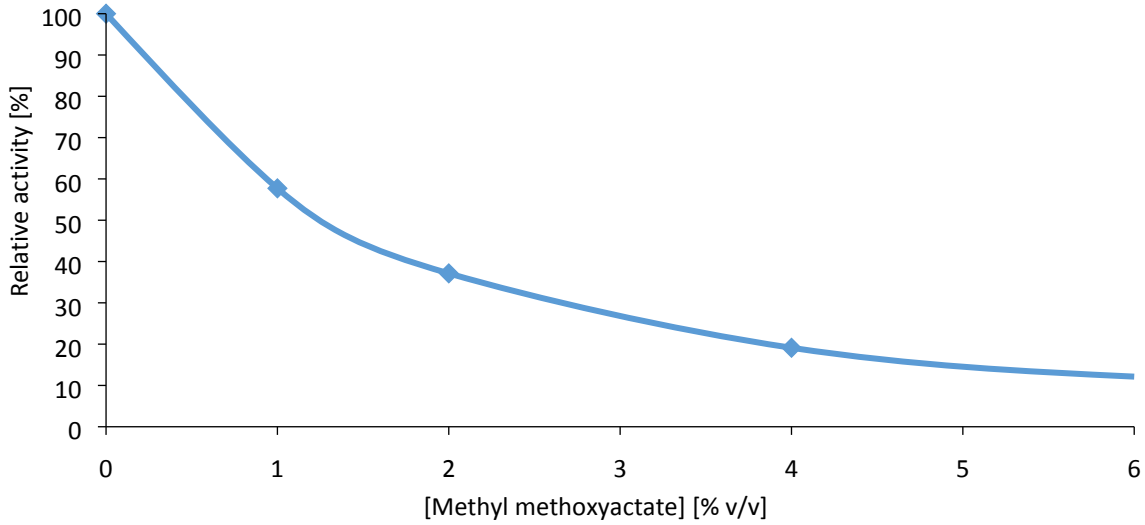
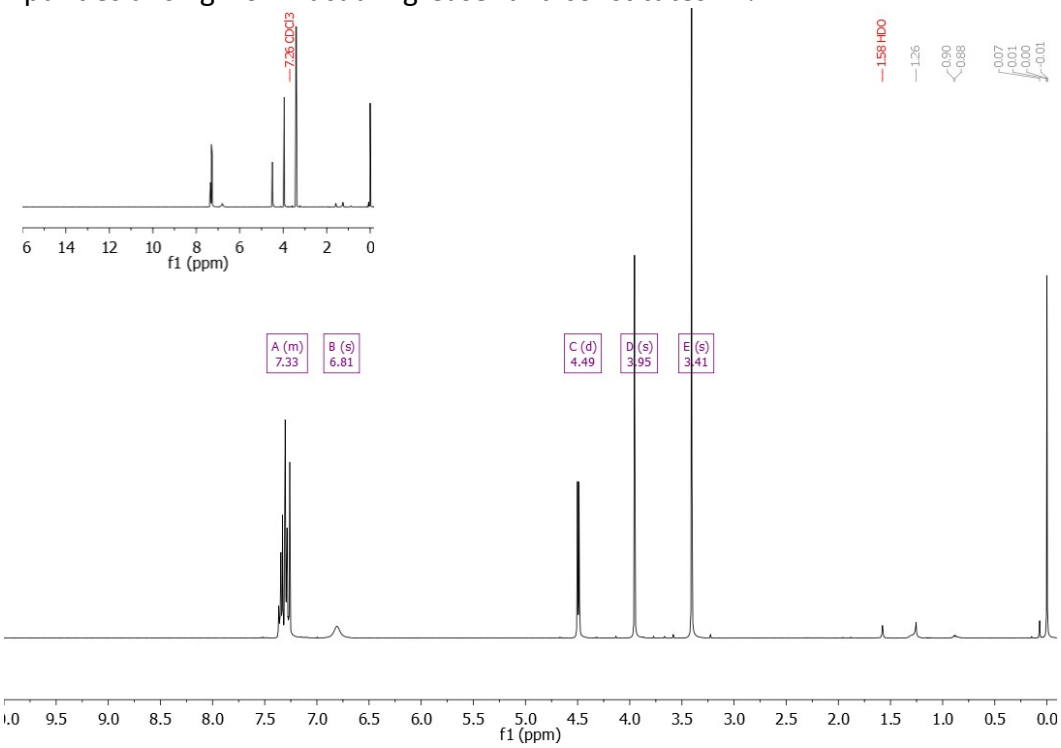


Fig. S2. *Sp*-ATA tolerance against increasing concentrations of methyl methoxyacetate. Enzyme activity was measured using the acetophenone assay.[2]

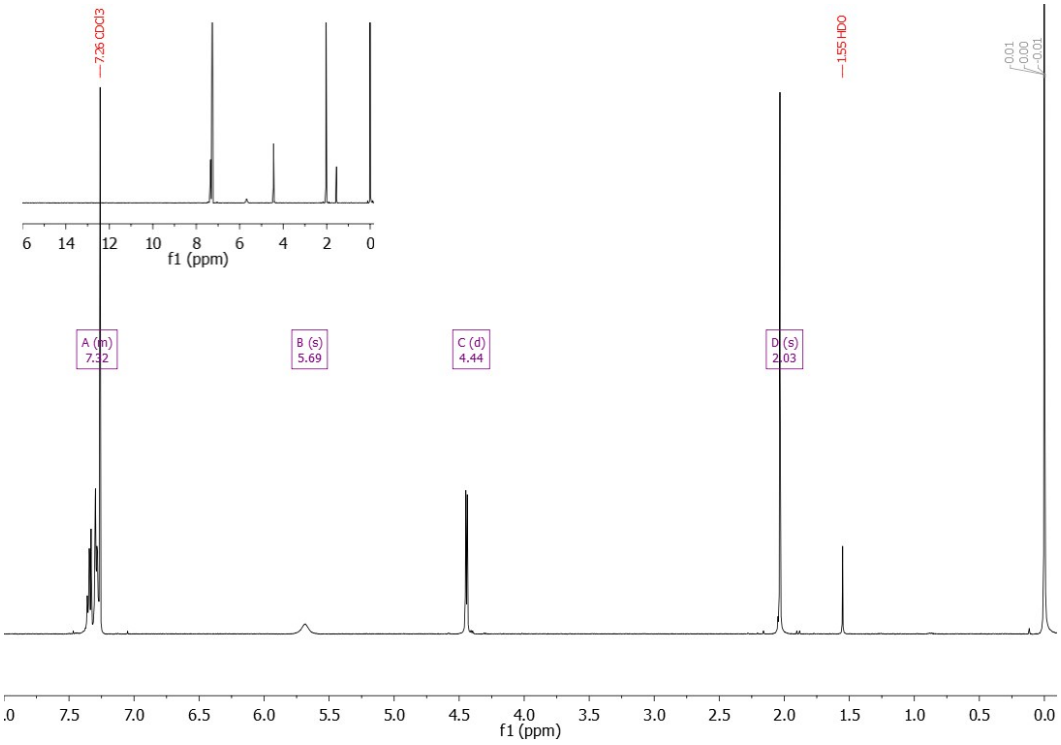
^{1}H-NMR of *N*-benzyl-2-methoxyacetamide (preparative synthesis)

δ_H (400 MHz, Chloroform-d) 7.40 – 7.24 (5 H, m), 6.81 (1 H, s), 4.49 (2 H, d, *J* 6.0), 3.95 (2 H, s), 3.41 (3 H, s). The chemical shifts at 1.26 (br s), 0.91-0.85 (m) and 0.07 (s) are consistent with impurities arising from vacuum grease[4] and constitutes ≤2%.



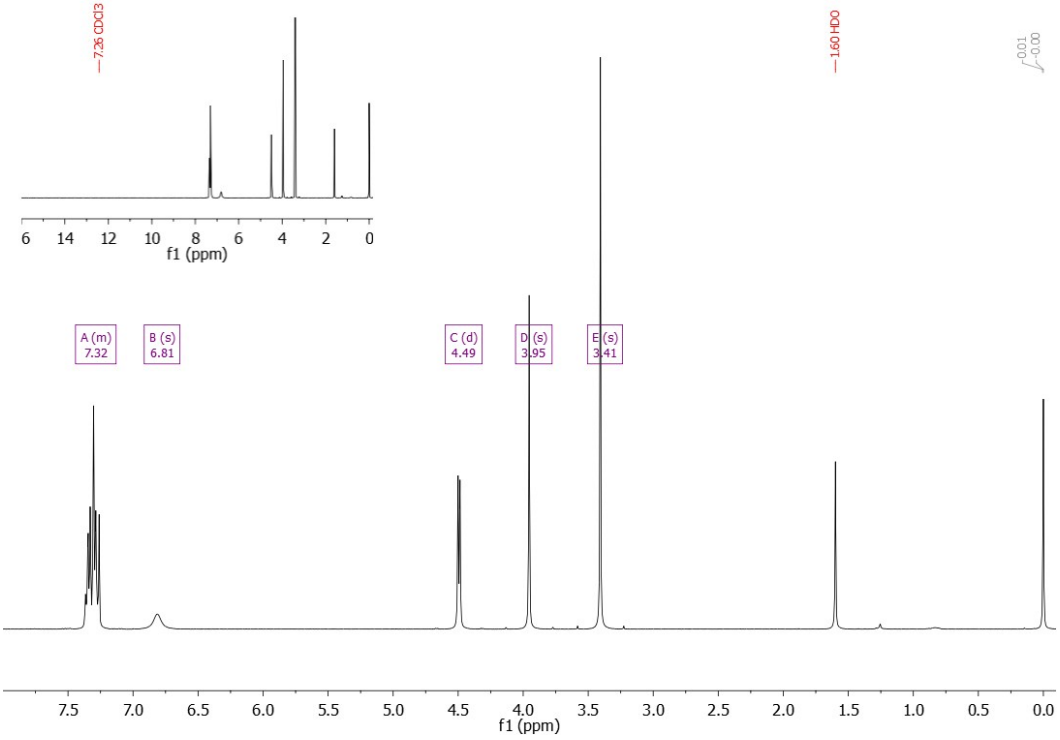
^{1}H-NMR of reference compound *N*-benzylacetamide

δ_H (500 MHz, Chloroform-d) 7.40 – 7.25 (5 H, m), 5.69 (1 H, s), 4.44 (2 H, d, *J* 5.7), 2.03 (3 H, s).



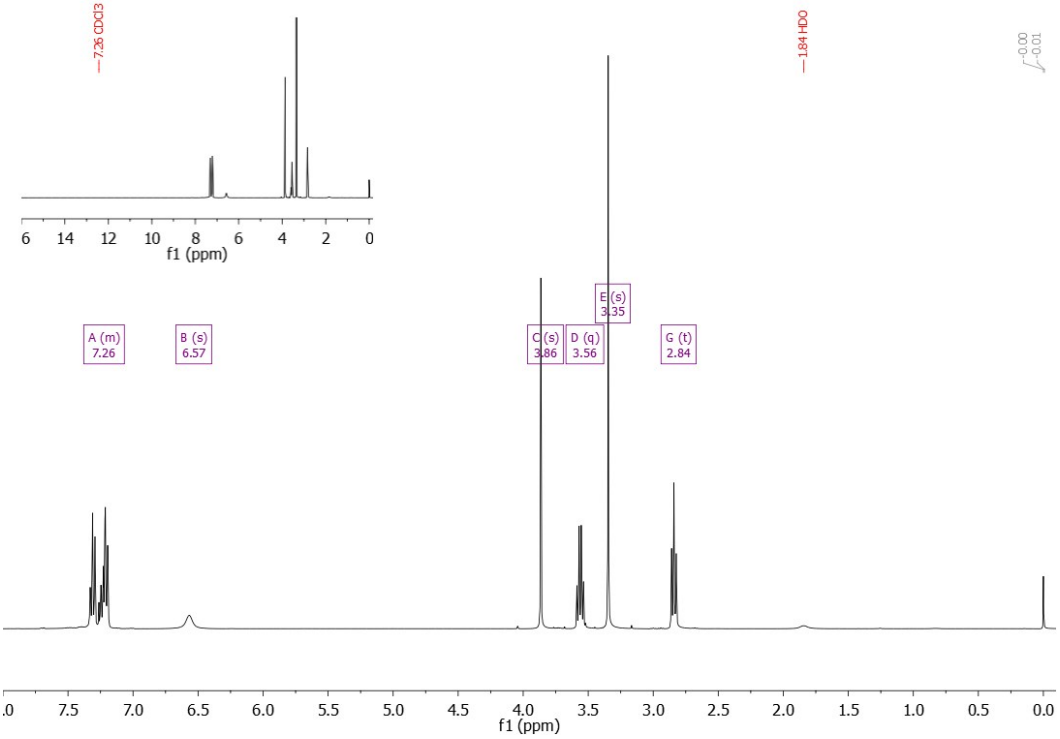
^{1}H-NMR of reference compound *N*-benzyl-2-methoxyacetamide

δ_H (400 MHz, Chloroform-d) 7.39 – 7.24 (5 H, m), 6.81 (1 H, s), 4.49 (2 H, d, *J* 5.9), 3.95 (2 H, s), 3.41 (3 H, s).



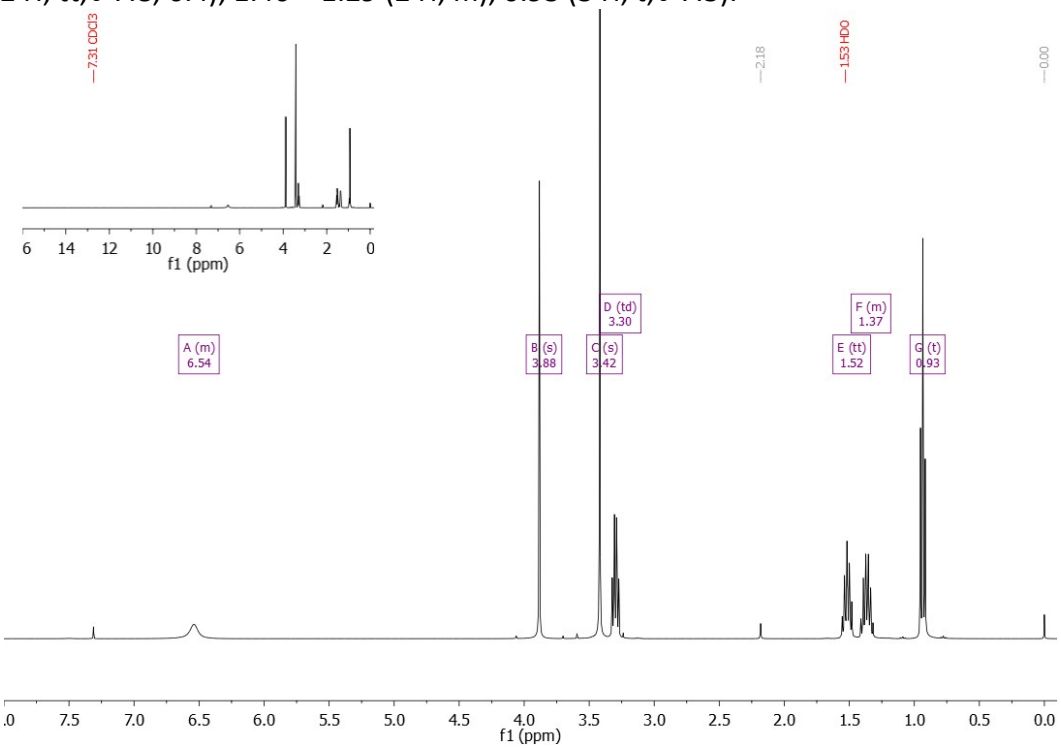
^{1}H-NMR of reference compound *N*-phenylethyl-2-methoxyacetamide

δ_H (400 MHz, Chloroform-d) 7.41 – 7.17 (5 H, m), 6.57 (1 H, s), 3.86 (2 H, s), 3.56 (2 H, q, *J* 6.8), 3.35 (3 H, s), 2.84 (2 H, t, *J* 7.2).



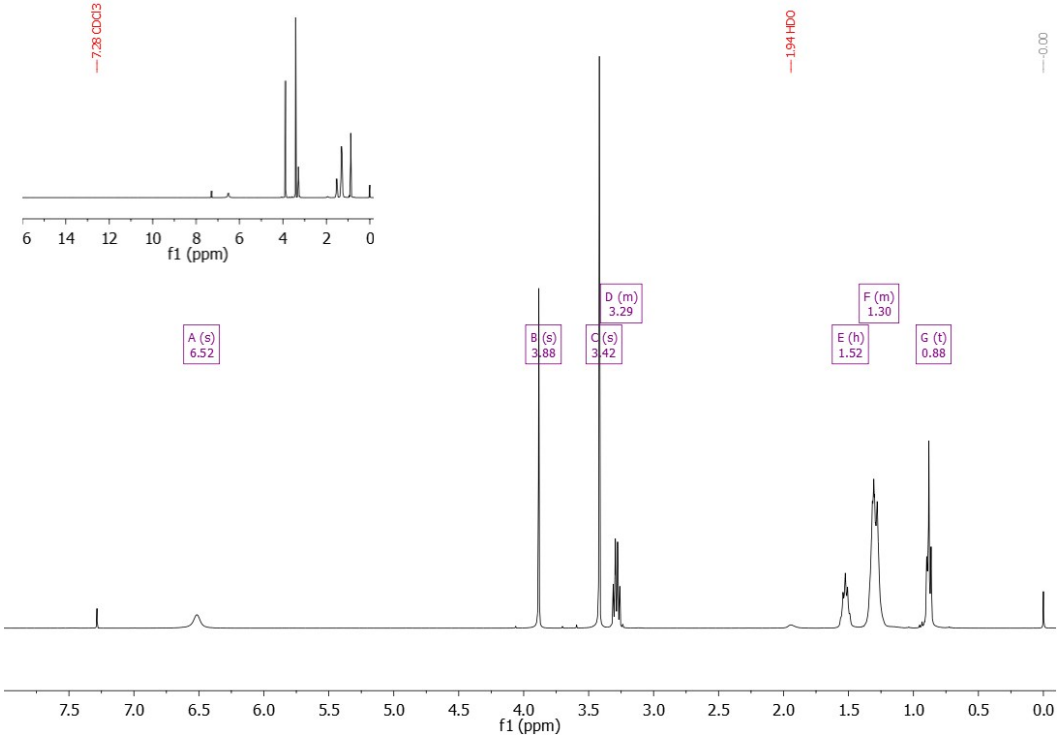
^{1}H-NMR of reference compound *N*-butyl-2-methoxyacetamide

δ_H (400 MHz, Chloroform-d) 6.54 (1 H, m), 3.88 (2 H, s), 3.42 (3 H, s), 3.30 (2 H, td, *J* 7.1, 5.9), 1.52 (2 H, tt, *J* 7.8, 6.4), 1.46 – 1.29 (2 H, m), 0.93 (3 H, t, *J* 7.3).



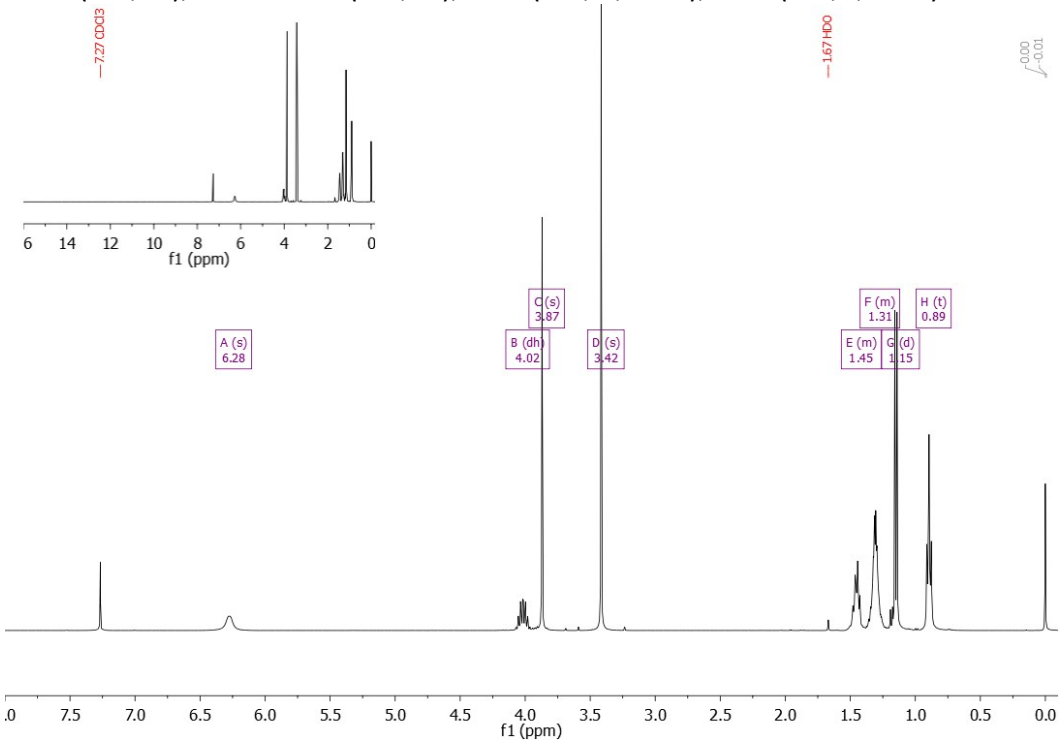
^{1}H-NMR of reference compound *N*-heptyl-2-methoxyacetamide

δ_H (400 MHz, Chloroform-d) 6.52 (1 H, s), 3.88 (2 H, s), 3.42 (3 H, s), 3.35 – 3.24 (2 H, m), 1.52 (2 H, h, *J* 7.1), 1.40 – 1.20 (8 H, m), 0.88 (3 H, t, *J* 6.8).



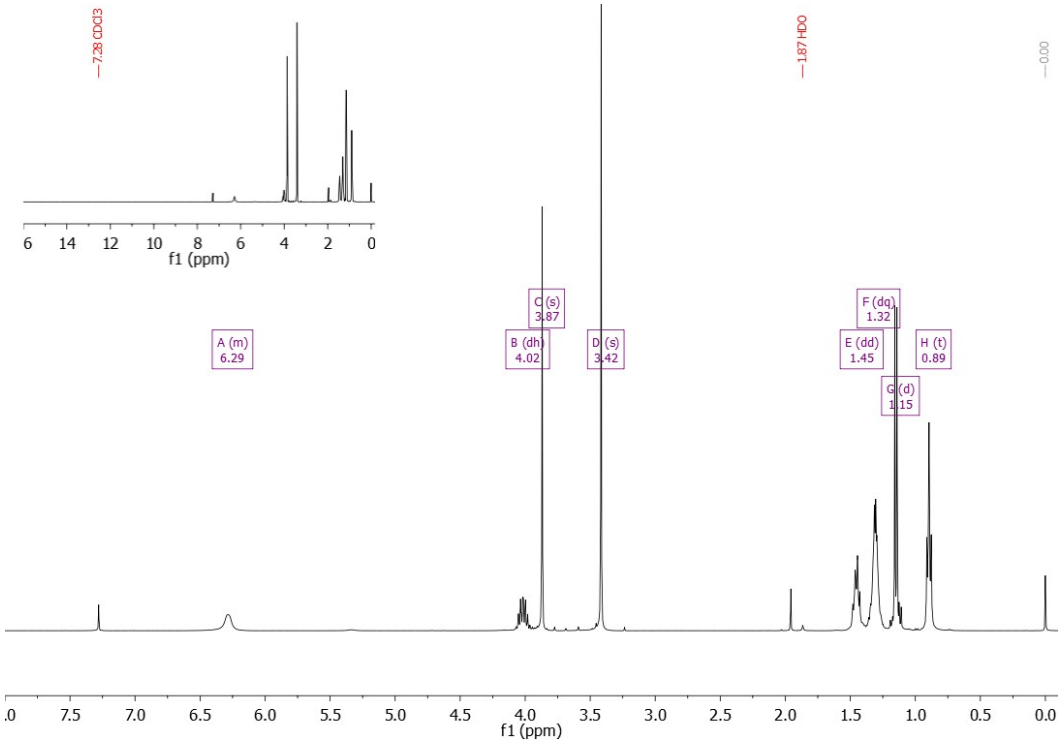
^{1}H-NMR of reference compound (*S*)-*N*-(hexan-2-yl)-2-methoxyacetamide

δ_H (400 MHz, Chloroform-d) 6.28 (1 H, s), 4.02 (1 H, dh, *J* 8.9, 6.7), 3.87 (2 H, s), 3.42 (3 H, s), 1.53 – 1.38 (2 H, m), 1.40 – 1.21 (4 H, m), 1.15 (3 H, d, *J* 6.6), 0.89 (3 H, t, *J* 6.8).



^{1}H-NMR of reference compound (*R*)-*N*-(hexan-2-yl)-2-methoxyacetamide

δ_H (400 MHz, Chloroform-d) 6.32 – 6.25 (1 H, m), 4.02 (1 H, dh, *J* 8.3, 6.6), 3.87 (2 H, s), 3.42 (3 H, s), 1.45 (2 H, dd, *J* 9.6, 4.6), 1.32 (4 H, dq, *J* 7.0, 4.8, 3.9), 1.15 (3 H, d, *J* 6.6), 0.89 (3 H, t, *J* 6.8).



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