Electronic Supplementary Material (ESI) for Analyst.

Rapid and sensitive monitoring of biocatalytic reactions using ion mobility mass spectrometry (IM-MS)

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SUPPORTING INFORMATION

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Supplementary figures

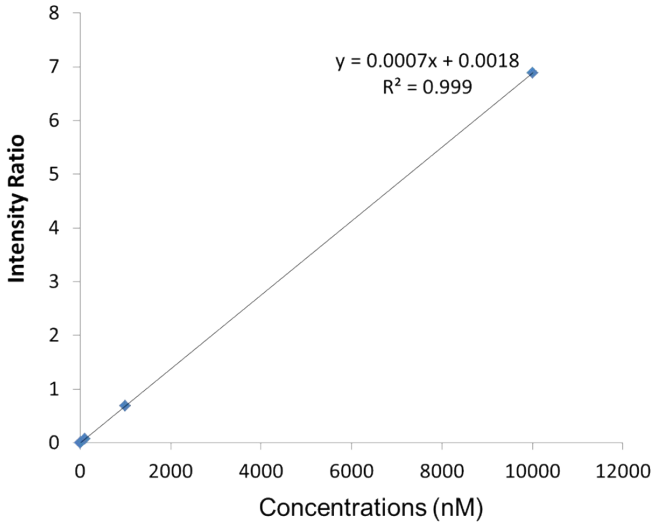


Figure S1. Standard curve of target amide **1A**, unlabeled form, spiked with leucine enkephalin as internal standard for calibrating the linear response of the nanoESI IM-MS.

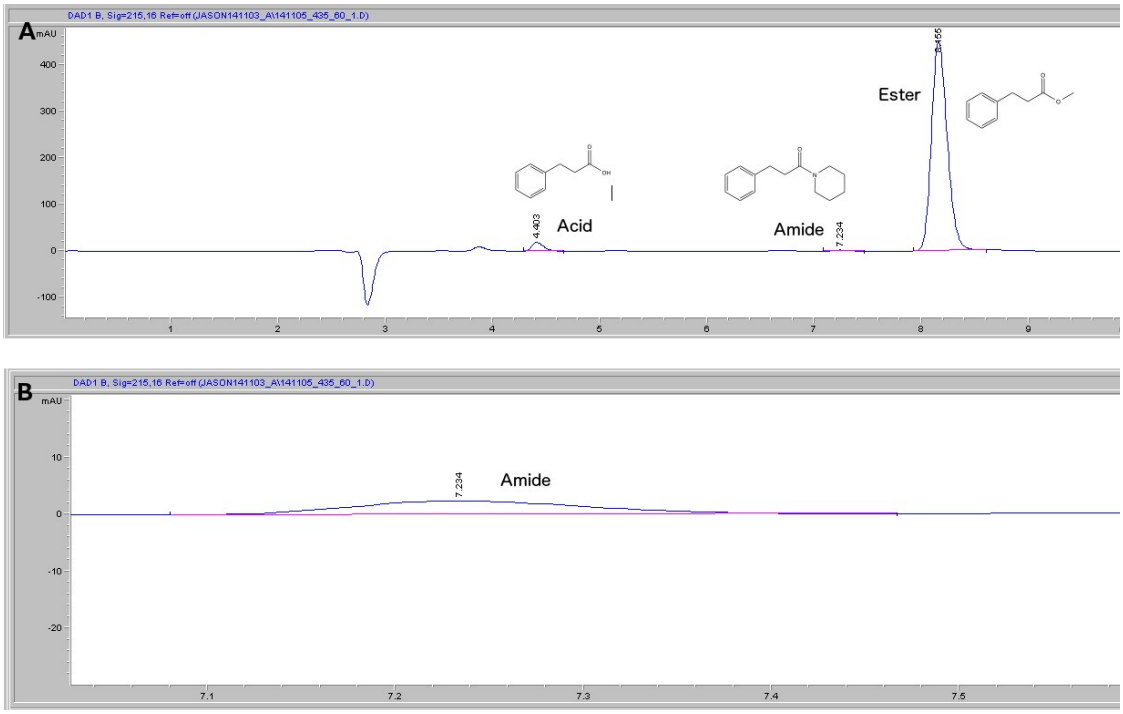
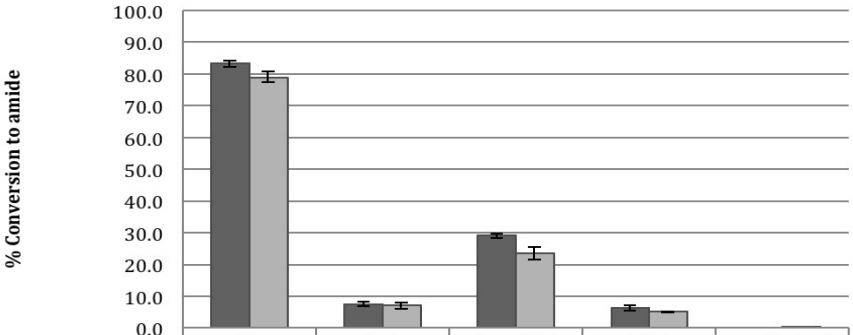


Figure S2. Limit of detection of the model reaction using HPLC. (A) Absorbance trace (λ = 215 nm) for the reaction of **1** with **A** after 1 h using CalB (N435) as a biocatalyst. Conversion corresponding to ~0.5 % is shown, and represents the visual limit of detection of **1A** (~5 μM). (B) Magnified view of the peak for **1A** from the same chromatogram.



	PSL	PFL	PFL5963	CalB	Neg Control
HPLC	83.2	7.6	29.1	6.2	0.0
nanoESI-IM-MS	78.9	7.1	23.5	5.1	0.0

Figure S3. Validation of the IM-MS approach for the screening of lipases for amidation activity. **1** and **A** were used as substrates (both 100 mM), and the reaction was conducted in TBME at 40°C for 24 h. Enzymes tested included commercially available lipases PSL (Lipase-TL, Meito Sangyo), PFL (Lipase AK, Amano), and CalB (N435, Novozymes), as well as the recombinantly expressed PFL5963. The negative control was based on a crude cell lysate mirroring the PFL5963 preparation though lacking the gene coding for the lipase. Amide formation was tracked by both HPLC (λ = 215 nm) and nanoESI IM-MS.

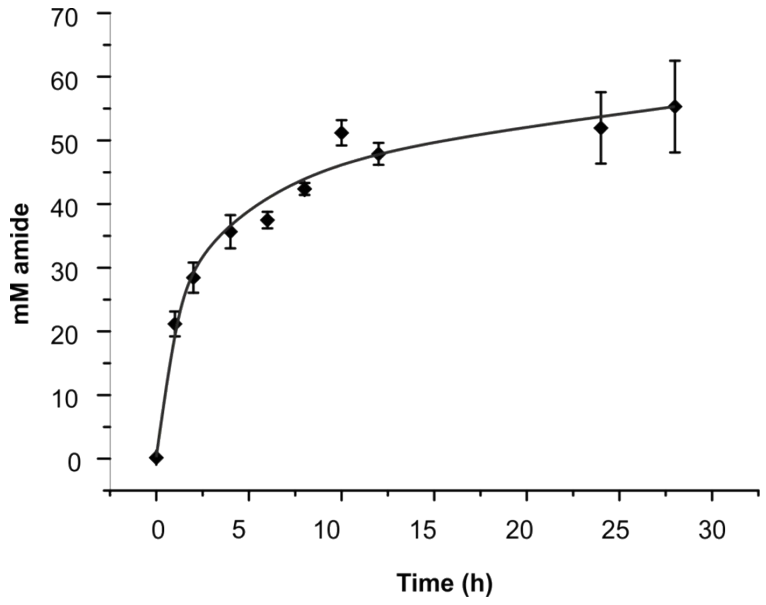


Figure S4. Monitoring of the time course of the PSL catalyzed reaction of **1** with **A** with nanoESI-IM MS.

Experimental section

Reagents and solvents

Unless stated elsewhere, all reagents and solvents were sourced from Sigma-Aldrich (Dorset, UK) or Fisher Scientific (Loughborough, UK) and used without further purification. Chemically competent *E. coli* BL21(DE3) cells were purchased from New England Biolabs Ltd. (Hitchin, UK).

Lipase catalyzed amidation reaction and sample preparation

The standard aminolysis reaction was performed using 30 mg of commercial PSL (Meito Sangyo, Japan), 50 mg of 4 Å molecular sieves (powdered form), added to 100 mM piperidine, 150 mM methyl 3-phenylpropanoate (Alfa Aesar), made up to 0.5 mL with *tert*-butylmethylether (TBME). Reactions were performed in 2 mL Eppendorf tubes, shaken at 1200 rpm at 40-50°C in a thermomixer for 24 h. TBME solvent was pre-dried by addition of granular 4 Å molecular sieves (pellet form). After the reaction, samples were prepared for analysis by centrifuging (17000 rcf for 5 min) to separate the solid and liquid phases. An aliquot (typically 10 µL) of the liquid phase was diluted stepwise into an 80% MeOH/H_2O solution (typically 1 in 100000 dilution). 200 µL of diluted sample was filtered (0.2 µm), loaded into a 96-well PCR microplate (Axygen Scientific), and sealed with aluminium self-adhesive foil (Thermo Scientific). Sample plates were stored at 4°C until analysis was performed.

For the substrate screening work, in a single reaction a panel of 5 esters (1 mM each) was reacted with the model reaction amine (piperidine **A**), and likewise 5 amines (1 mM each) were reacted with the model reaction ester (methyl 3-phenylpropionate **1**). Model substrates were used as a 1:1 mixture of deuterated and non-deuterated forms at a total concentration of 10 mM (5 mM of each). Each substrate screened was tested at 1 mM, both separately (five different 1 mL reactions), and in a one-pot format (a single 1 mL reaction) with 5 substrates (1 mM each), in all cases screened against model substrate in excess (5 mM/5 mM). To slow down the reaction, less enzyme was used in each reaction (6 mg), and the temperature lowered (30 °C). Reaction conditions were otherwise the same as mentioned above. For each one-pot reaction (both against model amine and model ester), a control reaction was also set up, lacking any enzyme. Samples were taken at time 0 h, 1 h, 2 h, 5 h and 23 h for each reaction tube and analysed.

P450 catalyzed oxidation reaction and sample preparation

The plasmid pET28a-P450-RhF (harbouring the P450-RhF gene) and an empty pET28a vector control were transformed into *E. coli* BL21(DE3) cells. The cells were grown at 37°C with 200 rpm shaking until optical density (OD_{600}) 0.8-1.0 was reached. Cells were cooled to 20°C prior to induction of protein expression by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.4 mM) and 5-aminolevulinic acid (5-ALA, 0.5 mM). The expression was continued for 20 h at 20°C. The cells expressing P450-RhF were harvested via centrifugation (4000 rpm, 4°C, 20 min) and re-suspended to 180 mg mL^{-1} (wet weight) in sodium phosphate buffer (50 mM, 100 mM KCl, 0.4% glycerol, pH 7.2). Diclofenac (1 mM) was added to the cell suspension (250 mL) and allowed to react in a shaking incubator for 24 h at 20°C with optimal shaking at 250 rpm. EtOAc (250 mL) was added to the reaction mixture, which was vortexed for 1 min and then centrifuged to aid phase

separation (13000 rpm, 4°C, 10 min). An aliquot of supernatant was filtered and transferred to a clean glass vial and stored at –20 °C for further analysis with nanoESI IM-MS.

nanoESI IM-MS apparatus

All the prepared samples were loaded onto a high-throughput, chip-based nanospray system, the Triversa Nanomate Robot from Advion Biosciences (Ithaca, NY, US), coupled to the Synapt HDMS G2-Si system equipped with travelling wave ion mobility spectrometry (TWIMS) (Waters, Manchester, UK). The pipette tip voltage was set to 1.5 kV and the gas pressure to 0.6 psi. Each sample was delivered for 3 min 10 s. Output contact closure was set to 10 s to trigger the mass spectrometer. Each pipette tip and chip nozzle was used only once for one injection avoiding any carryover effects. For the mass spectrometry, the parameters were set as follows: sample cone 40 V, source offset 30 V, source temperature 80°C, wave velocity 311 m/s and wave height 6.0 V for the trap, and 760 m/s, 40 V for the IMS, trap CE 4.0 V, transfer CE 1.0 V. Data was acquired over a range of m/z 50-600. By optimizing the travelling wave height and velocity, all the ion pack could be separated within the full drift time scale, which ensures efficient separation of gas phase ions. Mass spectrometry and drift time profiles were recorded for each biocatalytic reaction and viewed within Driftscope (Waters).

Assessing the linear response of nanoESI IM-MS

The linear response range of nanoESI IM-MS system was determined with amide **1A** spiked with leucine enkephalin (12.5 μg μL^{-1}) to ensure the right concentration range of testing samples. Series concentration solutions of **1A** were used (0, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM and 1 mM), the linear response curve was plotted by intensity ratio of peak area amide divided by the peak area of leucine encephalin against the amide concentrations. The system shows a good linear response in the concentration range of 10 nM - 10 μM (Figure S1), indicating that the intensity of the final product partly reflects the quantification information of the reaction. The linear response range also sets another possibility for the twin-substrate strategy: the initial ratio of light/heavy isotope labelled substrates can be adjusted within the linear response range freely, the ratio of light/heavy isotope labelled final product is in agreement with the substrate ratio in this case, and the substrate ratio is not limited to 1:1.

Assaying biocatalytic reactions with nanoESI IM-MS

Following direct injection of the sample, the total ion current (TIC) arrival time distribution (ATD) chromatography was monitored, and the theoretical mass to charge ratio of expected amides of both light isotope labeled and heavy isotope labeled amides were used to target specific sections of the TIC chromatography output. In the filtered spectra, only the components presented in both of the light and heavy forms with similar height peaks and identical drift time were checked with accumulated mass spectra. The mass shift of heavy isotope labeled final product must be in agreement with the possible isotope labeling sites. Both of the light and heavy isotope labeled amides are checked against their theoretical isotopic distributions. Considering the cross section distribution of potential amides, a total ion ATD from 1.5 to 4.0 ms was monitored (Figure 4a-b). Filtered ATD chromatographs with theoretical m/z ratios of light/heavy isotope labeled target amides were recorded. Substrates **1** and **A** were included in substrate panels as positive controls. The

detection of the control amide (**1A**) at DT = 2.5 ms in a 1:1 labeled/unlabeled m/z ratio was used to ensure the reliability of screening results.

Adaptation of method to accurate quantification and time course tracking of PSL catalyzed amidation

The twin-substrate strategy coupled with nanoESI-IM-MS has been verified above as a semi-quantitative analytical approach to tracking the model amidation reaction. In order to enable the accurate quantification for tracking the time course of PSL, the method was modified such that only D_{11}-piperidine was used in the reaction experiment. A known amount (typically representing 10% conversion) of the unlabeled amide (**1A**) was spiked into the diluent (80% MeOH) used in sample processing, to act as an internal standard. The ratio of the integrated peak area of heavy isotope labeled amide divided by the corresponding peak area of unlabeled amide (internal standard) was used to calculate the exact amount of amide formed under the biocatalysis of PSL. A time course of the PSL catalyzed model reaction over 28 hours was determined (Figure S4) and matched well to previously reported data derived from HPLC analysis. The data indicates that the method also provides us with an accurate quantification approach for monitoring the progress of a biocatalytic reaction by using direct infusion.

Lipase screening

To demonstrate the general applicability of the method to a range of enzyme sources, a lipase screen was conducted using the standard amidation reaction. Four lipases were tested: three commercial, and one recombinantly expressed in-house. Commercial lipases, PSL from *Pseudomonas stutzeri* (Lipase-TL, Meito Sangyo), PFL from *Pseudomonas fluorescens* (Lipase AK, Amano), and CalB from *Candida antarctica* (N435, Novozymes) were sourced as dry protein preparations and required no pre-processing. The recombinant lipase PFL5963 from *Pseudomonas fluorescens* was expressed recombinantly in *E. coli* cells, and required some processing to generate a suitable dry protein preparation for use in amidation experiments (see below). A negative control protein preparation was also constructed in parallel to PFL5963 that consisted of the same *E. coli* culturing process, though using the same expression vector that was lacking the lipase gene. For the screening, 30 mg of each protein preparation was added to 50 mg of 4 Å molecular sieves, and 500 µL of a reaction solution of 100 mM D_{11}-**A**, 100 mM **1** in TBME. This was incubated at 40°C for 24 h in 2 mL Eppendorf tubes. Upon sample processing for nanoESI IM-MS, 10 µL of the liquid phase were added to 990 µL of diluent (80% MeOH/H_2O spiked with unlabeled amide as internal standard for quantification) to give a 1 in 100 dilution. A subsequent 1 in 100 dilution with un-spiked diluent (only 80% MeOH/H_2O) was done, to give a 1 in 10000 final dilution that was then filtered and applied directly to nanoESI IM-MS.

Assay validation using HPLC-UVD/MS

In most cases, reactions were also measured by HPLC-UVD/MS on an Agilent 1100 Series instrument coupled to LC-MSD SL device. All analyses were carried out using a Kinetex C18 100A column (150 mm × 4.6 mm × 2.6 µm) with an isocratic flow of 37.5:62.5 MeCN/H_2O mixture and UVD monitoring (λ = 215 nm). MS was used to confirm the presence of the amide. Sample preparation was identical in post reaction processing until the dilution stage. A HPLC diluent solution was prepared by spiking a 50% MeOH/H_2O with 1,3-dimethoxybenzene (as HPLC internal standard) to give a 1 mM final solution. 10 µL of the centrifuged

biotransformation liquid phase was then added to 990 µL of the HPLC diluent solution to give a 1 in 100 dilution that was again centrifuged (13300 rpm, 10 min) before the HPLC injection. Baseline separation was achieved for all observed components. The internal standard was used to quantify the amide in the UVD trace.

Recombinant PFL5963 expression and protein preparation procedure

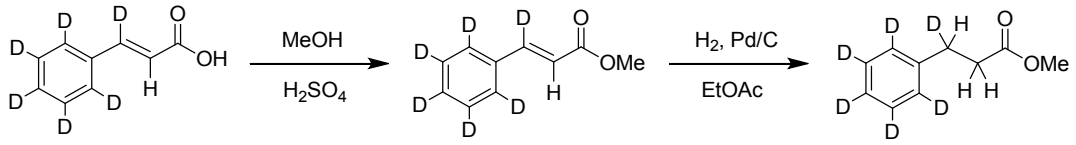
The structural gene coding for the lipase from *Pseudomonas fluorescens* JCM5963 (Uniprot ID: A9YY76) was synthesized (GeneArt, Life Technologies, Paisley, UK), and cloned into pET28b using the In-Fusion method (Clontech, Hampshire, UK), and the following primers:

Forward: 5'-aaatgggtcgggatcctcgccgcatgtcccaagagcttgc-3'

Reverse: 5'-cgagtgcggccgcaagcttcagaggccggcggccttgag-3'

The final construct featured an N-terminal His-tag followed by T7-Tag, thrombin site, then PFL5963. Optimal expression of active lipase was observed using auto-induction medium (trace-element-free super broth based, Formedium), cultured at 25°C for 5 days. Cells from 700 mL of media were harvested (4500 rcf, 4°C, 20 min), and the pellets re-suspended in 40 mL of 25 mM TrisCl, 300 mM NaCl, pH 8. Cells were lysed through incubation with lysozyme (Roche) followed by sonication, before being pelleted again (39000 rcf, 4°C, 20 min), and the pellet collected. The highest yields of active lipase per unit mass were recorded by removing the post sonication protein rich soluble fraction, which only featured low levels of soluble and active lipase. The insoluble pellet was then frozen with liquid nitrogen, and lyophilized (under vacuum) over night to give ~0.5 g of lipase protein preparation. This was ground into a fine powder and stored at 4°C.

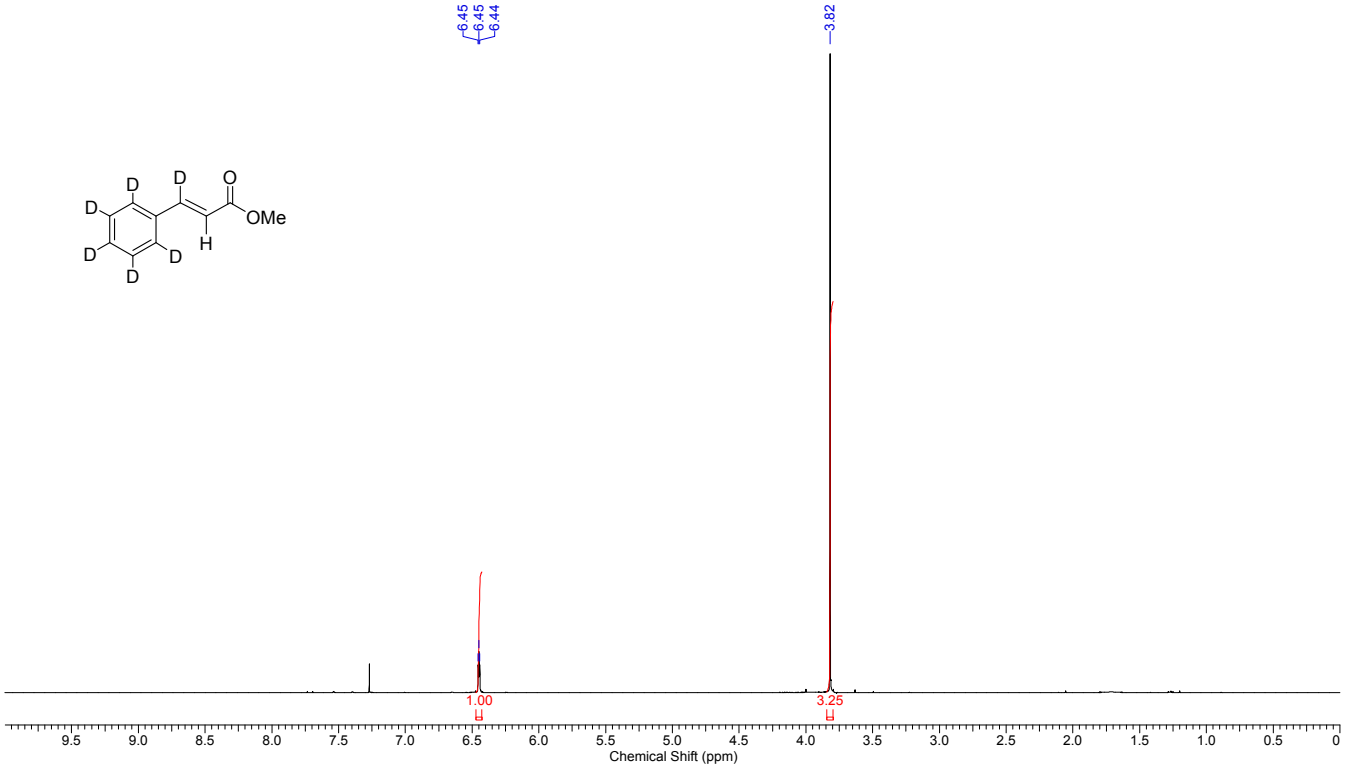
Synthesis of deuterium labelled ester 1

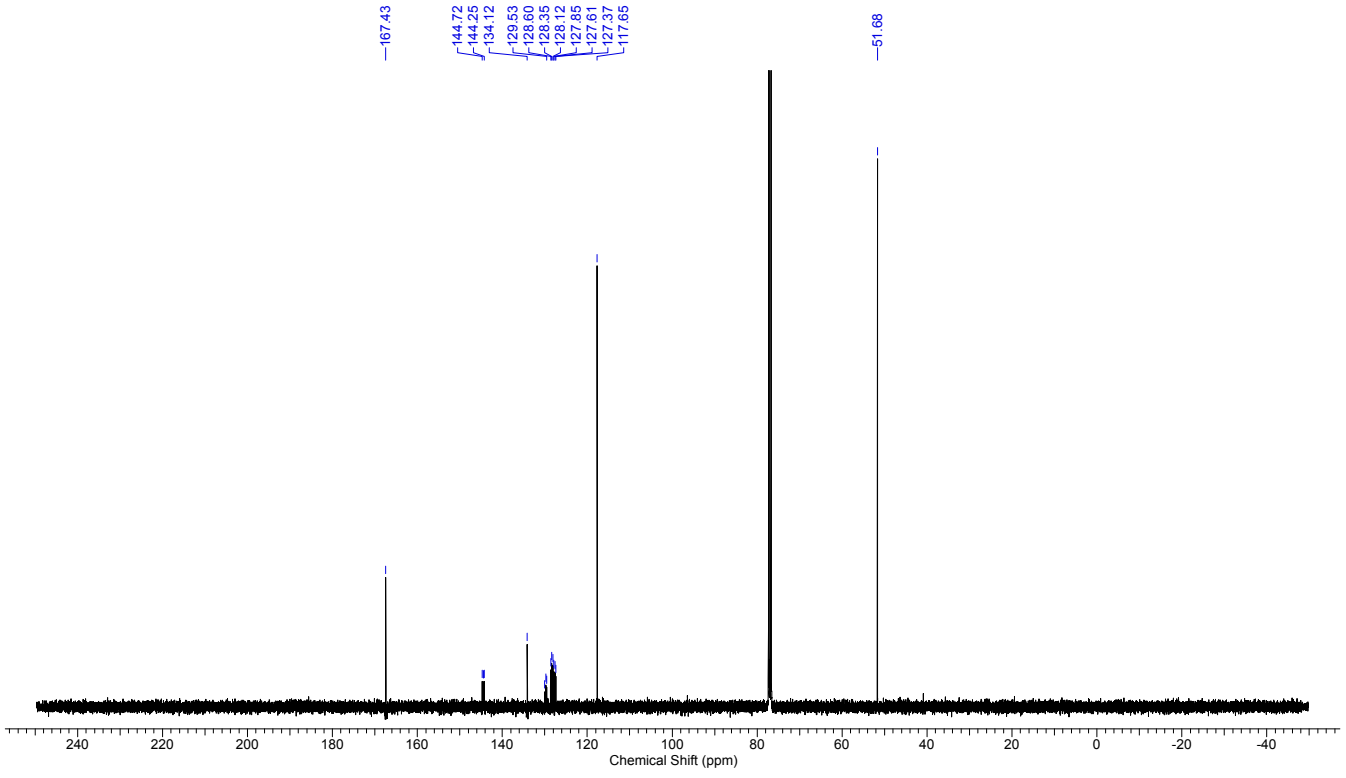


β,2,3,4,5,6-D_6-cinnamic acid (250 mg, 1.62 mmol, D incorporation: 98%) was dissolved in MeOH (5 mL) and H_2SO_4 (2 drops) was added. The mixture was stirred under reflux for 6 h, then most of the MeOH was removed under reduced pressure. The solution was diluted in EtOAc (5 mL), washed with $NaHCO_3$ solution (5 mL, 10% w/v) and extracted with EtOAc (3 × 5 mL). The combined organic phases were washed with brine (10 mL), dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to yield the corresponding methyl ester (267 mg, 1.59 mmol, 98% yield) as a crystalline colourless solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.45 (t, $^2J_{HD}$ = 2.3 Hz, 1H, C=C*H*), 3.82 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$): 167.5, 144.5 (t, J_{CD} = 23.5 Hz), 134.1, 129.8 (t, J_{CD} = 23.5 Hz), 128.4 (t, J_{CD} = 24.4 Hz), 127.6 (t, J_{CD} = 24.4 Hz), 117.7, 51.7.

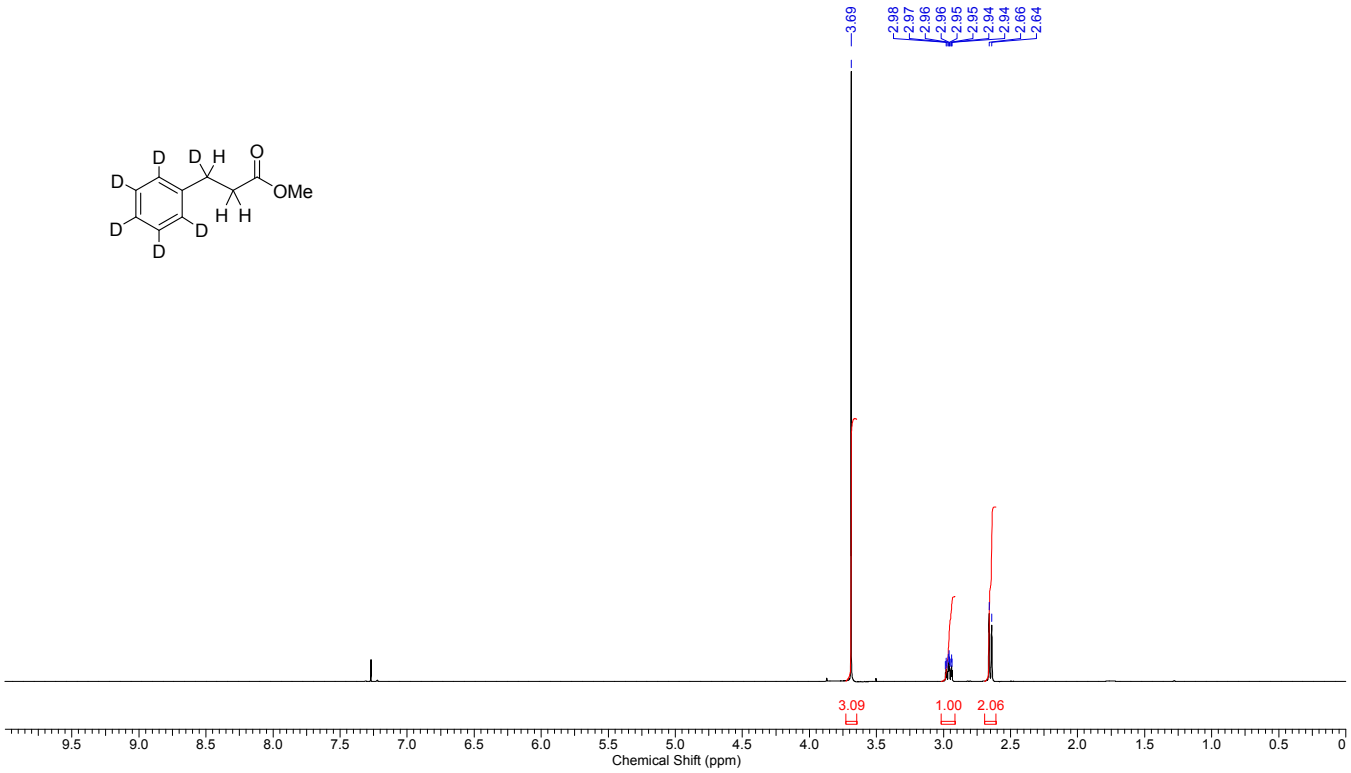
Methyl β,2,3,4,5,6-D_6-cinnamate (267 mg, 1.59 mmol) was dissolved in EtOAc (10 mL) and a catalytic amount of Pd/C 10% was added. The mixture was stirred under a H_2 atmosphere for 4 h at room temperature, filtered through a celite pad, dried over $MgSO_4$ and concentrated under reduced pressure to yield D_6-**1** (259 mg, 1.52 mmol, 96% yield) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.69 (s, 3H, OCH_3), 2.95 (tt, $^2J_{HD}$ = 2.1 Hz, ^3J = 7.9 Hz, 1H, C*H*D), 2.65 (d, ^3J = 7.9 Hz, 2H, CH_2). ^{13}C NMR (100 MHz, $CDCl_3$): 173.3, 140.2, 127.9 (t, J_{CD} = 24.3 Hz), 127.8 (t, J_{CD} = 23.9 Hz), 125.7 (t, J_{CD} = 24.3 Hz), 51.6, 35.6, 30.5 (t, J_{CD} = 19.7 Hz).

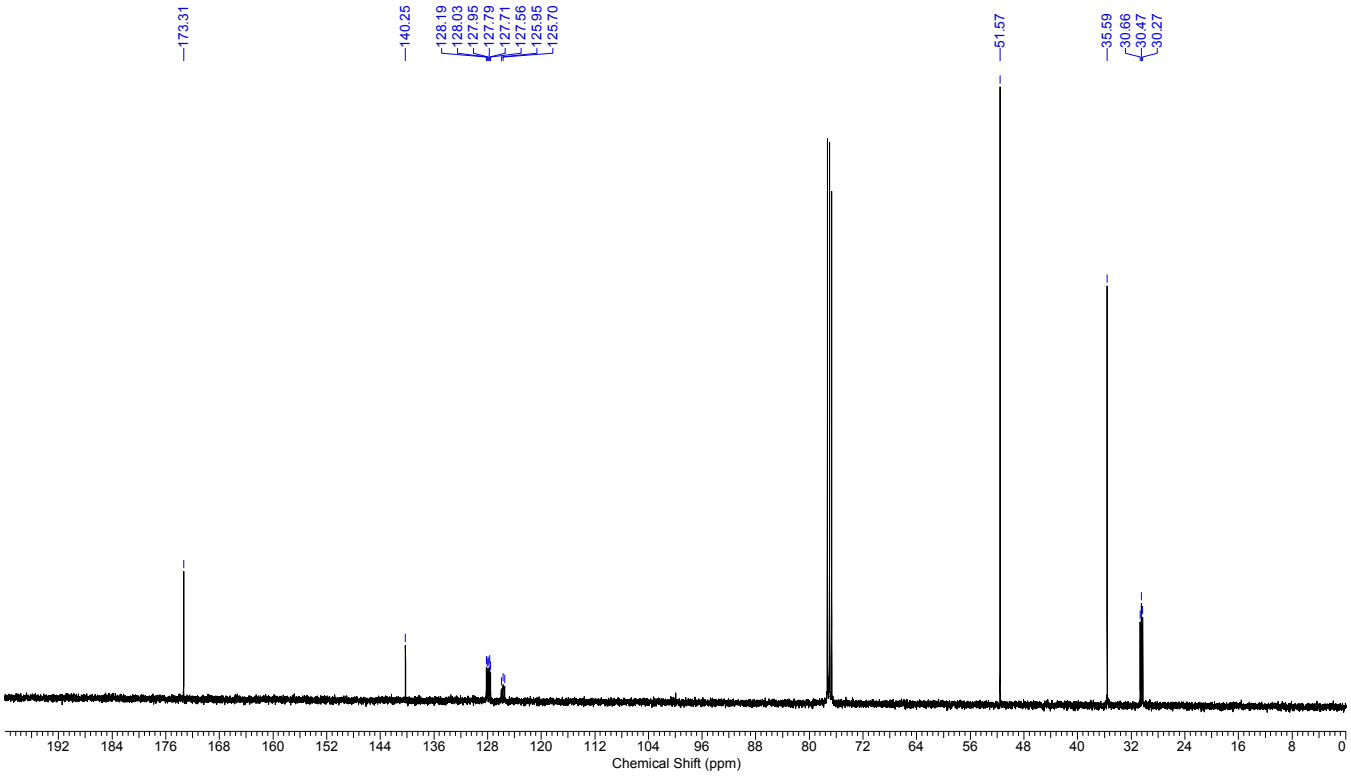
^{1}H and ^{13}C NMR spectra of methyl β,2,3,4,5,6-D_6-cinnamate





^{1}H and ^{13}C NMR spectra of methyl β,2,3,4,5,6-D_6-3-phenylpropanoate





Comparison of the aliphatic portion of the ^{1}H NMR spectra of commercial unlabelled methyl 3-phenylpropanoate **1** (a) and D_6-labelled methyl 3-phenylpropanoate **1** (b).