Electronic Supporting Information

Selective biocatalytic hydroxylation of unactivated methylene C-H bonds in cyclic alkyl substrates

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Experimental

General

General reagents, substrates and solvents were purchased from Sigma-Aldrich, Tokyo Chemical Industry (TCI), Alfa-Aesar and Acros-Organics. Antibiotics (ampicillin and kanamycin), isopropyl *β*-D-1-thiogalactopyranoside (IPTG), detergent (Tween 80) and dithiothreitol (DTT) were supplied by Astral Scientific (Australia).

The following media was used for bacterial growth and whole-cell turnovers (all per litre of media):

Lysogeny Broth (LB) [Yeast extract (5 g); Tryptone (10 g); NaCl (10 g)];

Trace elements solution [$FeCl_3$ ($6H_2O$) 16.7 g; Na_2EDTA 20.1 g; $CoCl_2$ ($6H_2O$) 0.25 g; $CaCl_2$ (H_2O) 0.74 g; $ZnSO_4$ ($7H_2O$) 0.18g; $CuSO_4$ ($5H_2O$) 0.10g; $MnSO_4$ ($4H_2O$) 0.132g];

E. coli minimal media (EMM) [K_2HPO_4 7 g; KH_2PO_4 3 g; $(NH_4)_2SO_4$ 1 g; $Na_3C_6H_5O_7$ (trisodium citrate) 0.5 g; $MgSO_4$ 0.1 g; 20% D-glucose 40 ml]

The antibiotics such as ampicillin (100 μg ml^{-1}) and kanamycin (30 μg ml^{-1}) were added to the growth media as required.

UV/Vis spectroscopy was performed on Varian Cary 60 or 5000 spectrophotometers, and these assays were maintained at 30 ± 0.5 ºC by an attached Peltier unit. The details of Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography (GC) analyses, methods, columns and instrument conditions are provided below (see Analysis of Turnovers section for details).

Enzyme Purification

Purification of CYP101B1

BL21 (DE3) *E. coli* competent cells were transformed with a plasmid vector (pET26; Merck Millipore) harbouring the CYP101B1 gene and grown on LB_{kan} plate.[1] A single colony was added to a flask of LB_{kan} (500 mL in each 2 L flask and a total of 3-4 L of media). These were grown at 37 ºC with shaking at 110 rpm for 10 hours. The temperature was decreased to 18 ºC, and 30 min later, ethanol (2% v/v) and benzyl alcohol (0.02% v/v) were added. Protein production was facilitated by adding 0.1 mM of IPTG from a 0.5 M stock solution. The culture was grown for a further 48 to 72 hours at 18 ºC and 90 rpm. The cell pellets were harvested by centrifugation (5000 *g*, 20 min at 4 ºC) and stored at -20 ºC before extraction. The pellets were resuspended in 200 mL Tris buffer (50 mM, pH 7.4) containing 1 mM DTT (Buffer T). The resuspended cells were lysed by sonication using an Autotune CV334 Ultrasonic Processor equipped with a standard probe (136 mm x 13 mm; Sonics and Materials, US). Pulses (40 x 20s on with 40 s intervals) were used to lyse the cells. The cell debris was discarded by

centrifugation (37 000 *g*, 20 min, 4 ºC). The protein containing supernatant was loaded onto a DEAE Sepharose column (XK50, 200 mm x 40 mm; GE Healthcare), and purified using a gradient from 100 to 250 mM KCl in buffer T at a flow rate of 6 mL min^{-1}. The red coloured protein containing fractions were combined and concentrated by ultrafiltration (10 kDa exclusion membrane). The concentrated protein was desalted by Sephadex G-25 medium grain column (200 mm x 40 mm) using Buffer T.

The desalted protein was concentrated and loaded onto a Source-Q ion-exchange column (XK26, 80 mm x 30 mm; GE Healthcare) linked to an AKTA Pure (GE Healthcare), and eluted using a linear gradient from 0 to 500 mM KCl in Buffer T. The fractions containing pure protein, which had an absorbance ratio $A_{419}/A_{280} > 1$, were combined and concentrated using ultrafiltration (10 kDa exclusion membrane). The concentrated protein was then centrifuged (7000 *g*, 10 min at 4 ºC) to remove solid debris. The protein was filtered using a 0.22 µm syringe filter after mixing with an equal volume of 80% glycerol and stored at -20 ºC. The extinction coefficient of CYP101B1 has been reported as ε_{417} = 113 mM^{-1} cm^{-1}, which was used to determine the concentration of the enzyme.[1, 2]

The ferredoxin reductase (ArR) and ferredoxin (Arx) were produced and purified as described previously using BL21 (DE3) *E. coli* cells and the pET26 vector containing the ArR gene and a pRSFDuet plasmid harbouring the gene encoding the ferredoxin (Arx).[1]

Substrate Binding Assays

CYP101B1 was diluted to ~ 3 µM with 50 mM Tris buffer (pH 7.4). The UV-Vis spectrum was recorded from 700 nm to 250 nm. An aliquot of the substrate (1 µL from 100 mM stock solution in ethanol) was added to 500 µL of the enzyme solution, and the spectrum recorded. Further aliquots of the substrate were added until the shift from low-spin (420 nm) to high-spin (390 nm) did not change. The percentage of high-spin (HS) state shift induced by the substrate was estimated by comparison with the spectra of camphor-free and camphor-bound $P450_{cam}$ (CYP101A1).

Dissociation constants (K_d) were determined to measure how tightly the substrates bind to the enzymes. The P450 enzymes were diluted to ~1 µM with 50 mM Tris buffer pH 7.4. The UV-Vis spectrum of 2.5 mL of the enzyme was recorded between 600 nm to 300 nm, and the spectrophotometer was baselined. Successive aliquots of the substrate (cumulative volumes from 0.5 to 10 µL from 1 mM stock solution in ethanol/DMSO) were added, and the different spectra were recorded. Further aliquots (cumulative 1 to 10 µL) of the substrate from 10 mM stock solution were then added. Finally, aliquots from 100 mM stock solution were added until the peak to trough absorbance difference remained constant. The peak to trough absorbance differences were plotted against the substrate concentration to determine the dissociation constant of the substrate by fitting to a hyperbolic function (Eqn. 1):

$$\Delta A = \frac{\Delta A_{max} \times [S]}{K_d + [S]} \qquad \textbf{Eqn. 1}$$

Where ΔA represents the peak to trough absorbance difference, ΔA_{max} is the maximum absorbance difference, [S] represents substrate concentration, and K_d is the dissociation constant.

If substrates exhibited tight binding, defined as having a dissociation constant less than five times that of the enzyme concentration (K_d < 5[E]), the data was instead fitted to the tight binding quadratic equation (Morrison) (Eqn. 2).[3]

$$\frac{\Delta A}{\Delta A_{max}} = \frac{([E]+[S]+K_d) - \sqrt{\{([E]+[S]+K_d)^2 - 4[E][S]\}}}{2[E]} \qquad \textbf{Eqn. 2}$$

where ΔA is the peak-to-trough absorbance difference, ΔA_{max} represents the maximum absorbance difference, [S] is the substrate concentration and [E] represents the enzyme concentration

In vitro NADH Turnover Assays

NADH turnover/consumption rates were measured by preparing a 1.2 mL mixture containing 0.5 µM of P450 enzyme, 5 µM ferredoxin (Arx), 1 µM ferredoxin reductase (ArR), 12 µL bovine liver catalase (10 mg mL^{-1}) and oxygenated 50 mM Tris buffer, pH 7.4, in a cuvette. This was allowed to reach 30 ºC, and NADH was added to a concentration ~ 320 µM (A_{340}~ 2). The absorbance was monitored at 340 nm using a UV-Vis spectrophotometer. After one minute, 0.5 - 1 mM of the substrate from a 100 mM stock solution in ethanol was then added and mixed by inversion (the amount of substrate added was dependent on the substrate solubility). The NADH oxidation rate was calculated by recording the gradient of the absorbance at 340 nm versus time and using the extinction coefficient of NADH, ε_{340} = 6.22 mM^{-1} cm^{-1}.

Whole-cell Biotransformations

The DNA vector pETDuet harbouring the genes of the ferredoxin (Arx) and ferredoxin reductase (ArR), and the pRSFDuet plasmid containing the CYP101B1 and Arx genes (as described previously), were transformed together into BL21 (DE3) *E. coli* competent cells [1]. The transformed cells were grown on a $LB_{kan/amp}$ plate overnight at 37 ºC. A single colony from the plate was added to $LB_{kan/amp}$ (500 mL) media and allowed to grow for another 10 hours at 37 ºC with shaking (120 rpm). Protein production was induced as described in the previous sections and the culture was grown at 18 ºC and 90 rpm overnight. The cell pellets (~12 g of cell wet weight per litre; P450 concentration approx. 0.65 µM) were harvested from the growth media by centrifugation (5000 *g*, 20 min at 4 ºC), and cells were resuspended in 1 L *E. coli* minimal media (EMM).[1, 4]

The resuspended cells (200-250 mL) were split into 2 L baffled flasks. Substrate (0.5 to 1 mM; the amount depended on substrate solubility) was added and the flasks were shaken at 30 ºC and 150 rpm for 4 h. Further aliquots of the substrate (0.5 to 2 mM) were added along with additional glucose (2 mL from 20% solution) and phosphate-buffered saline (PBS, 2.5% v/v). The reactions were continued overnight before the cells were removed from the supernatant by centrifugation (5000 *g*, 20 min, 4 ºC). Products were extracted from the supernatant by liquid-liquid extraction using ethyl acetate or dichloromethane (3 x 100 mL), washed with brine and dried with anhydrous magnesium sulphate. The extracted products were isolated by silica column chromatography using a gradient of 90:10 to 50:50 hexane to ethyl acetate, using a 5% increase in every 50 to 100 mL. The purified metabolites were characterised by NMR in deuterated chloroform. NMR spectra were acquired on an Agilent DD2 or a Varian Inova spectrometer, operating at 500 or 600 MHz for ^{1}H and 126 or 151 MHz for ^{13}C. A combination of ^{1}H, ^{13}C, COSY, HSQC, HMBC and ROESY experiments were used to determine the structure of the metabolites.

Analysis of turnovers

The turnovers were analysed via GC (two columns, wax and chiral) and GC-MS (DB5ms column) to assess product formation versus impurities and to ensure the separation of all the metabolites. Co-elution experiments were used to identify the products generated in turnovers with authentic standards using GC and GC-MS. Control experiments were performed (turnovers with no NADH and or enzyme added to determine peaks arising from impurities). If standards were not available, the metabolites were synthesised in larger yield using a whole-cell oxidation system for characterisation. For GC and GC-MS analysis, 990 µL of the reaction mixture was taken and mixed with 10 µL of internal standard (20 mM *p*-cresol in ethanol). The mixture was then extracted with 400 µL of ethyl acetate. The organic extracts were analysed directly by GC or GC-MS.

The ratio of total area of product(s) and internal standard was used to calculate the product concentration. Metabolites were calibrated using the authentic product standard or isomers of the product or substrates where authentic products not available. These standards, at different concentrations (10 µM to 200 µM), were combined with an internal standard (200 µM) as described above for sample preparation. These were then analysed via GC or GC-MS using the same method as for the turnovers. Product and internal standard area ratios were plotted against the concentrations to create calibration curves which were used to measure the amount of metabolites generated in the turnovers. To calculate the productive use of reducing equivalents (coupling efficiency), the concentration of the product formed in the turnover was divided by the concentration of NADH that was added (calculated from the absorbance at 340 nm). The proportion of product formed per NADH

molecule oxidised (the coupling efficiency) was used with the NADH oxidation rate to give the product formation rate.

GC Instrument Operating Conditions

GC-MS	Shimadzu GC-17A instrument attached to a QP5050A MS detector or Shimadzu GC-2010 coupled to a GC-MS-QP2010S detector (EI positive ion mode)
Column	ZebronDB-5 MS fused silica column (30 m x 0.25 mm x 0.25 µm)
Column carrier gas	Helium; flow rate 1.3 ml min^{-1}
Injector and interface temperature	250 °C and 280 °C
Method for cyclic alkanes, ketones and esters	140 °C (hold 3 min), 10 °C/ min to 220 °C (hold 7 min.)
Method for cyclic alkanes (C6-C8)	120 °C (hold 3 min), 10 °C/ min to 220 °C (hold 7 min.)
GC	Shimadzu Tracera GC coupled to Barrier Discharge Ionization Detector (BID)
Column	Supelcowax column (Supelco, 30 m x 0.32 mm x 0.25 µm)
Column carrier gas	Helium and flow rate 1.3 ml min^{-1}
Injector and BID temperatures	250 °C
Method for cyclic alkanes, ketones and esters	120 °C (hold 3 min), 5 °C min^{-1} to 220 °C (hold 3 min)
Method for cyclic alkanes (C6-C8)	80 °C (hold 3 min), 5 °C min^{-1} to 220 °C (hold 3 min)
Chiral GC	Shimadzu Tracera GC coupled to Barrier Discharge Ionization Detector (BID)
Chiral Column	RT®- BDEXse chiral silica column (Restek; 30 m x 0.32 mm x 0.25 µm)
Column carrier gas	Helium and flow rate 3 ml min^{-1}
Injector and BID temperatures	230 °C
Method for cyclic alkanes, ketones and esters	100 °C (hold 3 min), 5 °C min^{-1} to 200 °C (hold 3 min)
Method for cyclic alkanes (C6-C8)	80 °C (hold 3 min), 5 °C min^{-1} to 200 °C (hold 3 min)

The total turnover number (TTN) was determined with the best substrates to monitor the efficiency and activity of the enzyme over an extended period. These were determined using a ArR:Arx:CYP101B1 concentration ratio of 1:10:1. The CYP enzyme (0.1 µM) was combined with ArR (0.1 mM), Arx (1 µM), 2 mM substrate and 4 mM NADH (theoretical maximum 20,000 turnovers). Oxygenated 50 mM Tris buffer, pH 7.4, was added to the mixture to a final volume of 1.2

mL. The turnover mixture was kept at room temperature for 2 hours before extraction with 400 µL of ethyl acetate. The organic extracts were analysed directly by GC/GC-MS.

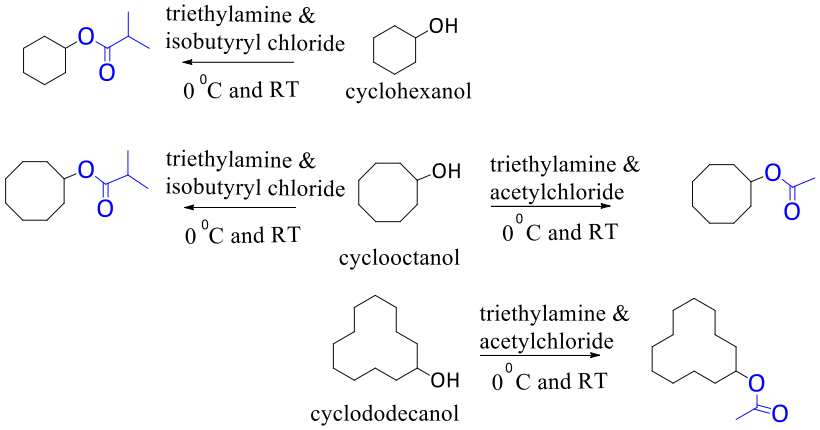
Table of conversions and isolated purified yield of the major product metabolites.

Substrate	**substrate added**	**conversion**	**Isolated yield of purified product mg**
cyclododecanol	2 mM	>90%	Total 25 mg (3 products one major and two minor) see Figure S3 d and S5 ii
cyclodecanone	3 mM	50%	1-oxabicyclo[5.3.1]undecan-1-ol and 5-hydroxycyclodecanone, total (22 mg) 1-oxabicyclo[6.3.1]undecan-1-ol and 6-hydroxycyclodecanone total (12 mg)
cycloundecanone	2 mM	60%	5-hydroxycycloundecanone (10 mg), 6-hydroxycycloundecanone (15 mg)
cyclododecanone	2 mM	55%	7-hydroxycyclododecanone (27 mg)
cyclohexyl acetate	4 mM	50%	4-*trans*-hydroxycyclohexyl acetate (29 mg)
cyclohexyl butyrate	4 mM	60%	*trans*-4-hydroxycyclohexyl butyrate (26 mg)
cyclohexyl isobutyrate	4 mM	45%	*trans*-4-hydroxycyclohexyl isobutyrate (32 mg)
methyl cyclohexylacetate	4 mM	55%	methyl (*trans*-4-hydroxycyclohexyl)acetate (32 mg)
ethyl cyclohexylacetate	4 mM	40%	ethyl (*trans*-4-hydroxycyclohexyl)acetate (24 mg)
cyclooctyl acetate	4 mM	35%	*trans*-5-hydroxycyclooctyl acetate (31 mg)
cyclooctyl isobutyrate	3 mM	40%	*trans*-5-hydroxycyclooctyl isobutyrate (25 mg)
cyclododecyl acetate	3 mM	40%	*trans*-7-hydroxycyclododecyl acetate (21 mg)

The whole -turnovers were performed on 150 ml scale. The conversion was estimated based on GC/GC-MS analysis of the turnover upon completion. The concentration of substrate added to the reaction over the course of the turnover is provided. Note that these turnovers were performed as described in the in the experimental with low cell density and minimal optimisation.

Synthesis of Ester Substrates

The cyclic alcohols substrates (cyclohexanol, cyclooctanol and cyclododecanol) (~0.5 g, 1.0 eq.) were dissolved in dichloromethane (~20 mL), and trimethylamine (~2.0 eq.) was added at 0 °C and stirred for 5.0 minutes. Isobutyryl chloride (~1.2 eq.) or acetyl chloride (~1.2 eq.) was then added dropwise at 0 °C, and the reaction was subsequently stirred at room temperature for 3.0 hours.

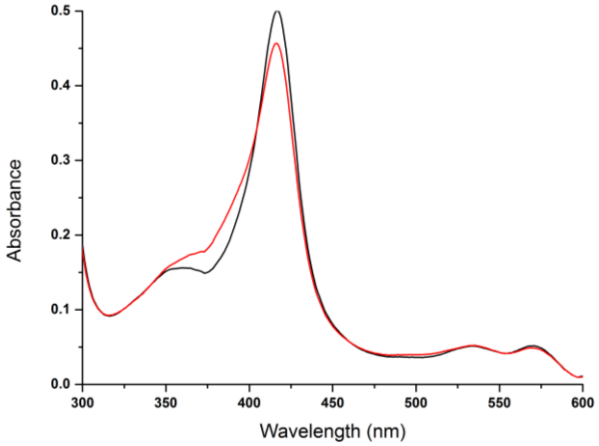


The synthesis of cyclohexyl isobutyrate, cycooctyl acetate, cyclooctyl isobutyrate and cyclododecyl acetate.

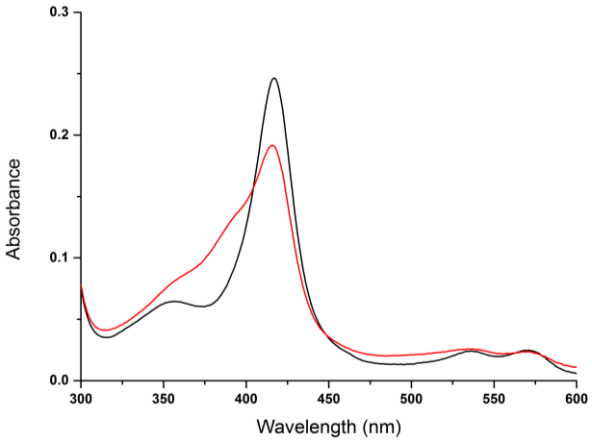
On completion, the reaction mixtures were washed with a saturated sodium bicarbonate solution. The organic layer was isolated and washed with brine solution, dried over anhydrous Na_2SO_4 or $MgSO_4$, filtered and concentrated under vacuum to obtain the crude product. The products were separated by silica chromatography using a 5% ethyl acetate/petroleum benzene mixture, and the acetate/isobutyrate products were obtained as colourless oils. The synthesised substrates were characterised by NMR (Figure S6).

Substrates	**(NMR)**
cyclohexyl isobutyrate Conversion and Yield (55% and 273 mg)	^{1}H NMR (500 MHz, $CDCl_3$) δ 4.82-4.66 (m, 1H, H1), 2.52 (*septet*, *J* = 7.0 Hz, 1H, H8), 1.88-1.66 (m, 10H), 1.16 (d, *J* = 7.0 Hz, 6H, 3xH9 & 3xH10).
cyclooctyl acetate Conversion and Yield (48% and 240 mg)	^{1}H NMR (500 MHz, $CDCl_3$) δ 4.95-4.85 (m, 1H, H1), 1.99 (s, 3H, 3xH10), 1.83-1.42 (m, 14H).
cyclooctyl isobutyrate Conversion and Yield (62% and 310 mg)	^{1}H NMR (500 MHz, $CDCl_3$) δ 4.99-4.84 (m, 1H, H1), 2.49 (*septet*, *J* = 7.0 Hz, 1H, H10), 1.85-1.43 (m, 14H), 1.16 (d, *J* = 7.0 Hz, 6H, 3xH11 & 3xH12).
cyclododecyl acetate Conversion and Yield (50% and 270 mg)	^{1}H NMR (500 MHz, $CDCl_3$) δ 5.05-4.95 (m, 1H, H1), 2.03 (s, 3H, 3xH14), 1.76-1.63 & 1.53-1.26 (m, 21H).

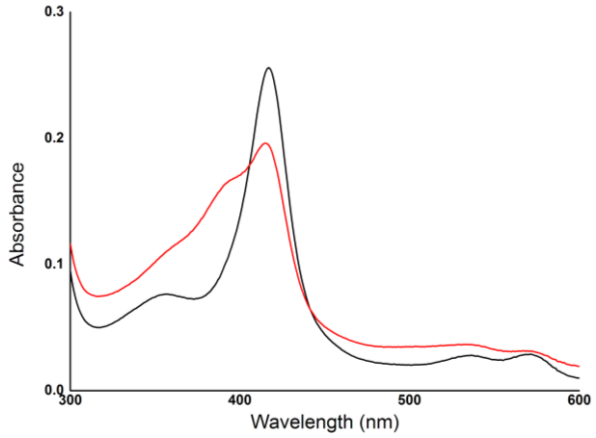
Figure S1 The spin-state shifts induced in CYP101B1 upon addition of selected substrates (black substrate free CYP101B1; red substrate bound enzyme).



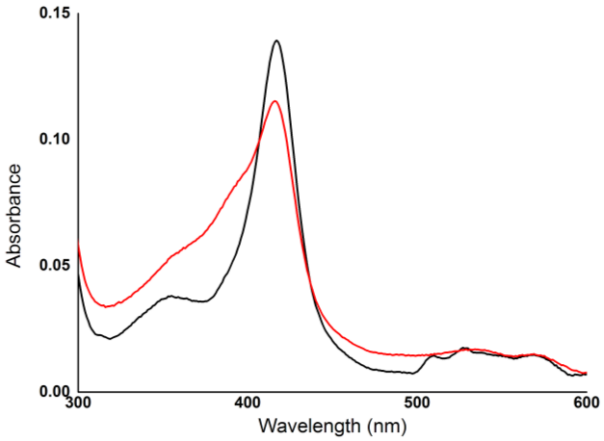
cyclohexane



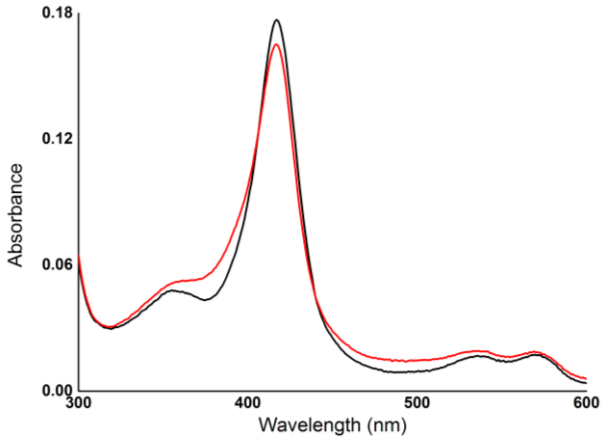
cyclooctane



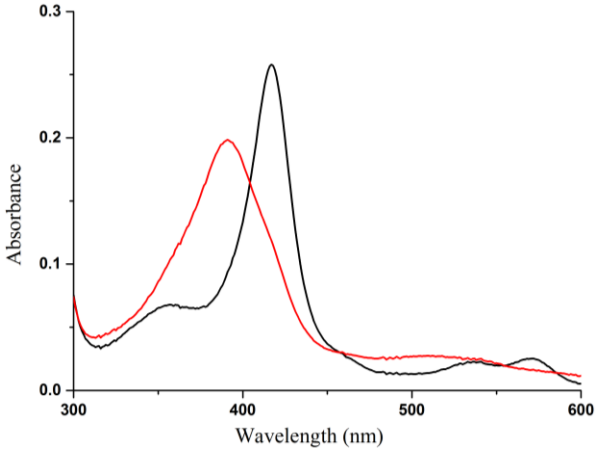
cyclodecane



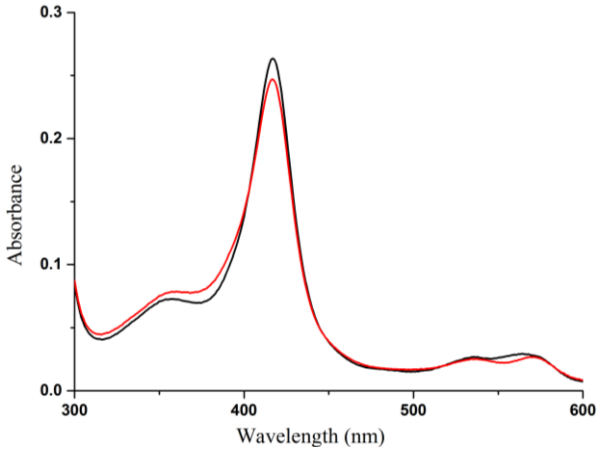
cyclododecane



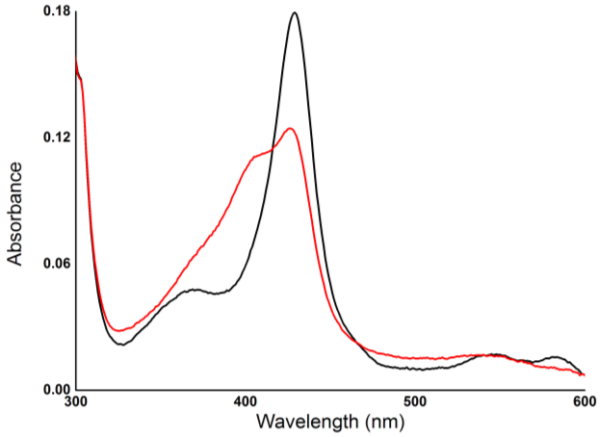
cyclooctanol



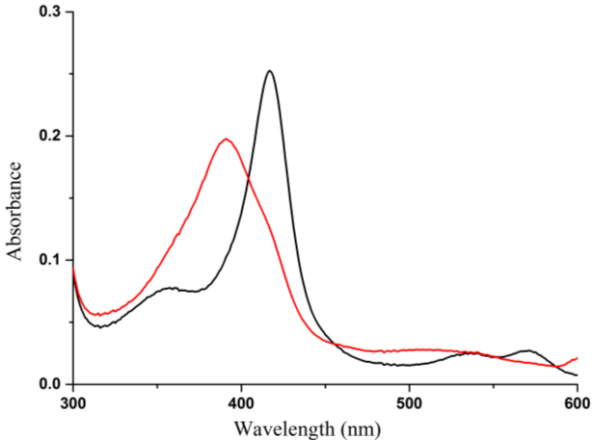
cyclododecanol



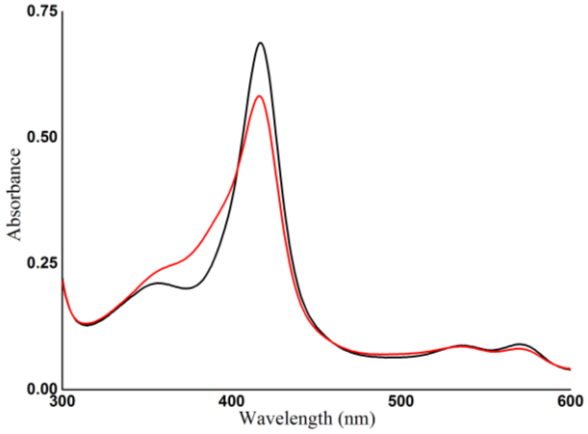
cyclooctanone



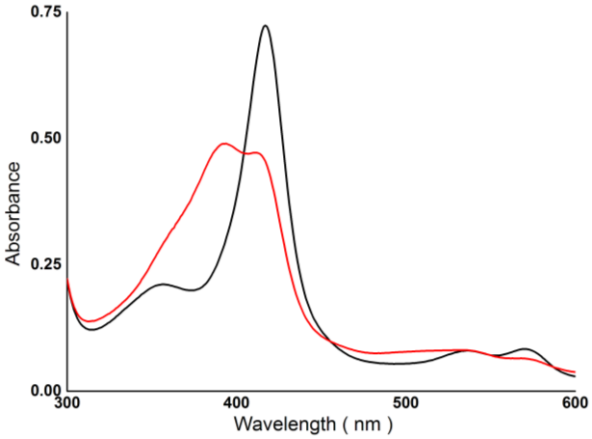
cyclodecanone



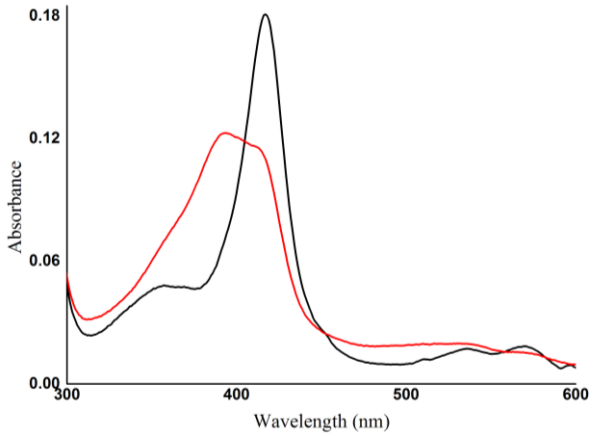
Cyclododecanone



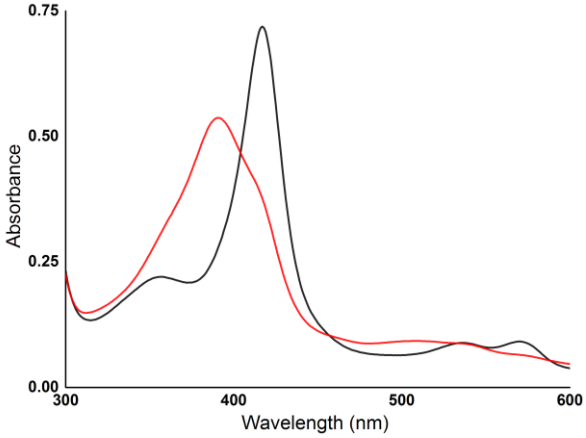
cyclohexylacetate



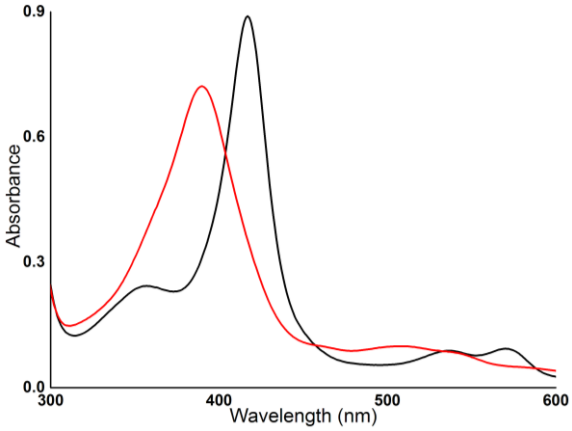
cyclohexyl butyrate



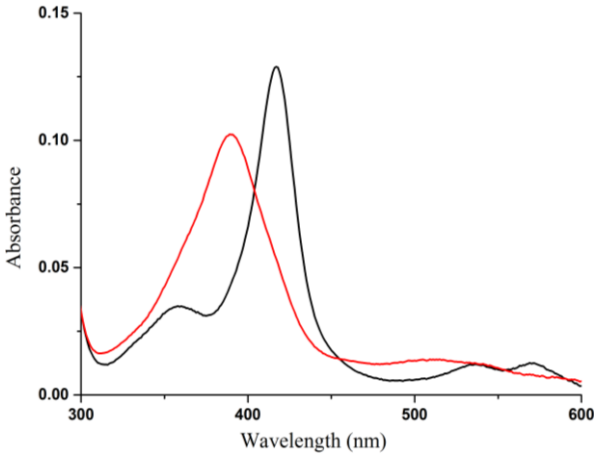
cyclohexyl isobutyrate



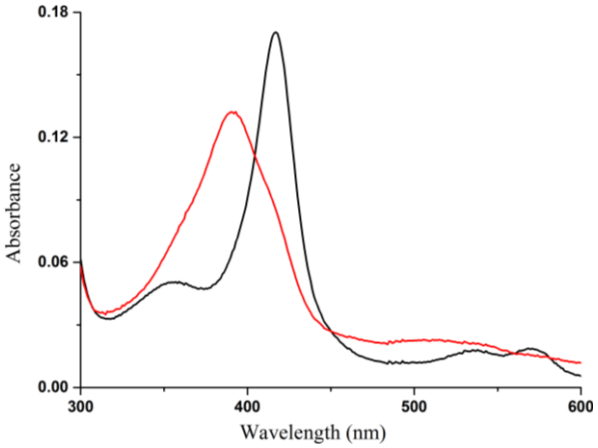
ethyl cyclohexylacetate



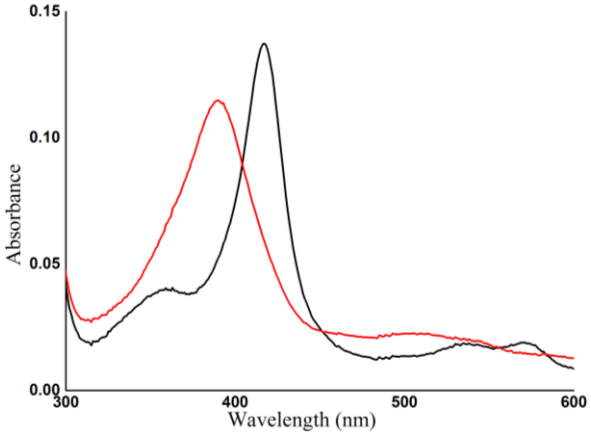
methyl cyclohexylacetate



cyclooctyl acetate

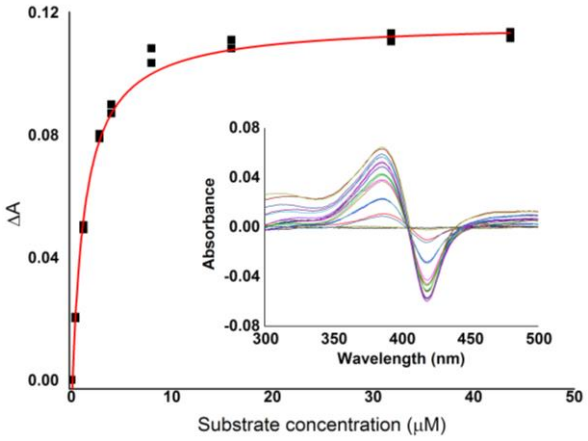


cyclooctyl isobutyrate



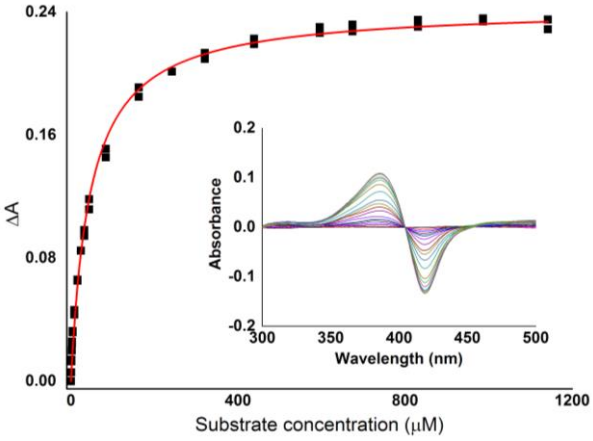
cyclododecyl acetate

Figure S2 Analysis of the dissociation constants of selected substrates with CYP101B1. The inset shows a typical titration of the substrate and CYP101B1. The concentration of enzyme used in the analysis and the dissociation constant (*Kd*) is provided in brackets.



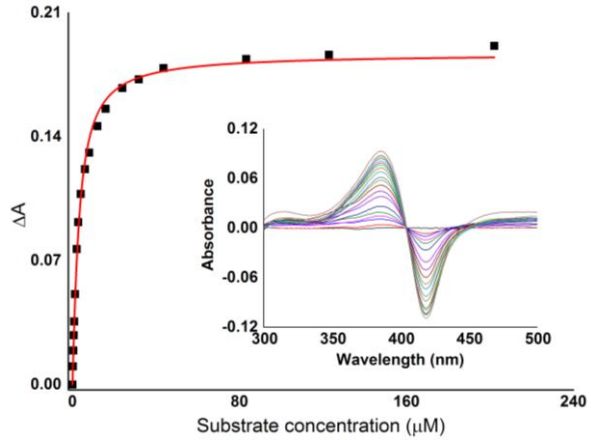
cyclodecane

(1.2 μM CYP101B1/K_d 0.88 μM)



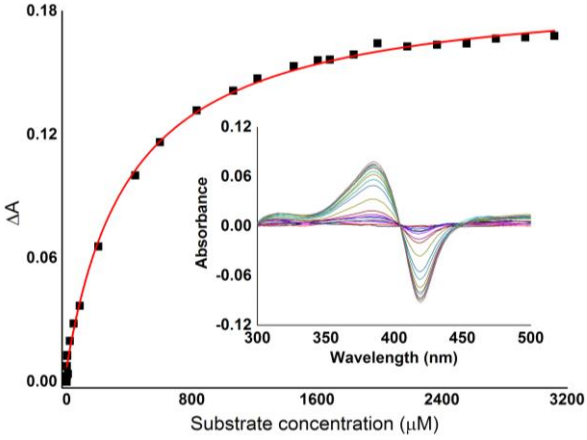
cyclodecanone

(2.4 μM CYP101B1/K_d 46μM)



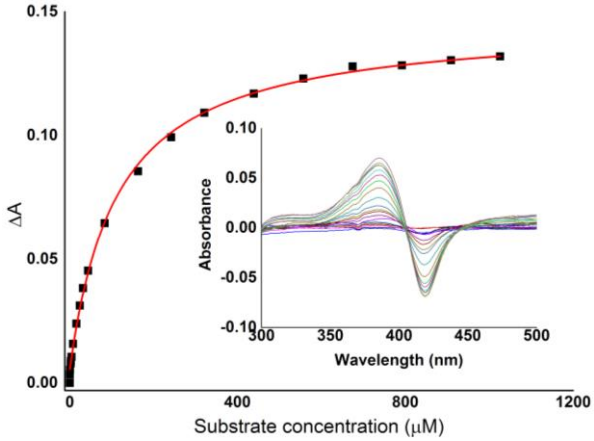
cyclododecanone

(1.6 μM CYP101B1/K_d 2.0 μM)



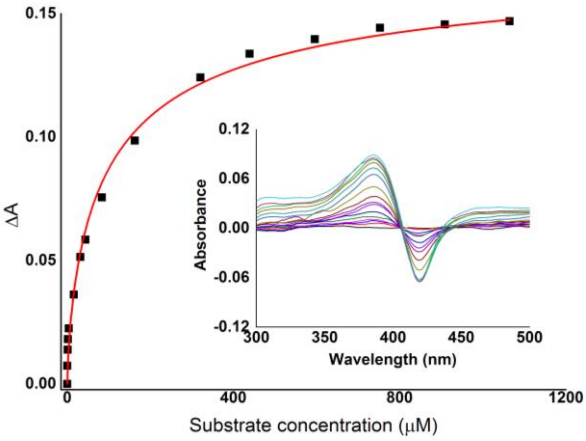
cyclohexyl acetate

(1.7 μM CYP101B1/K_d 540 μM)



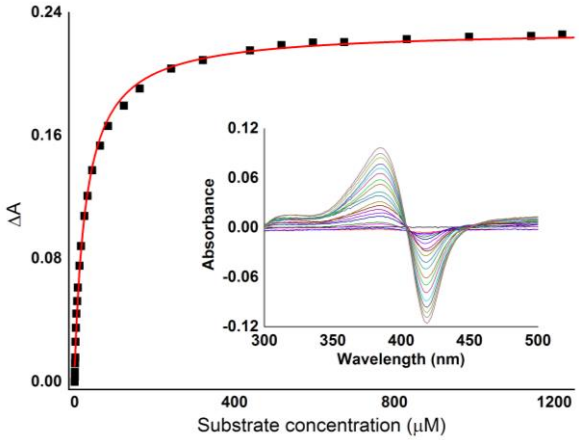
cyclohexyl butyrate

(1.3 μM CYP101B1/K_d 110 μM)

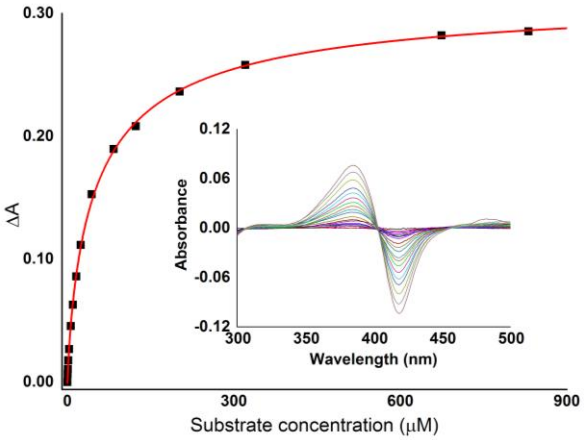


cyclohexyl isobutyrate

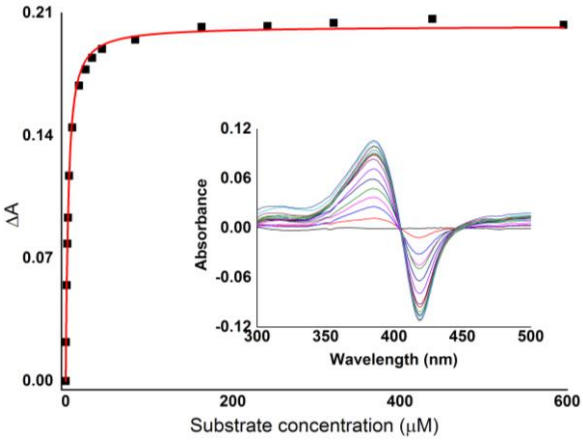
(1.7 μM CYP101B1/K_d 61 μM)



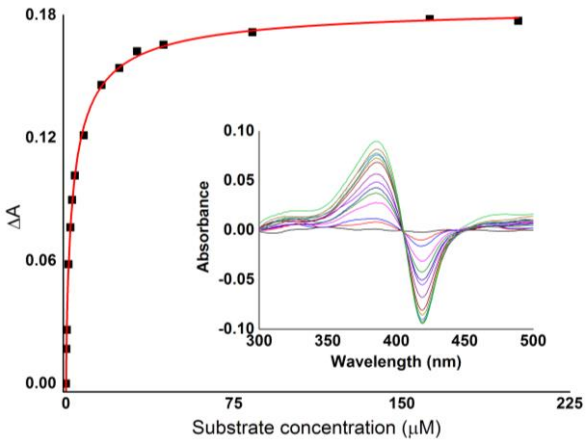
methyl cyclohexylacetate
(1.70 μM CYP101B1/K_d 31 μM)



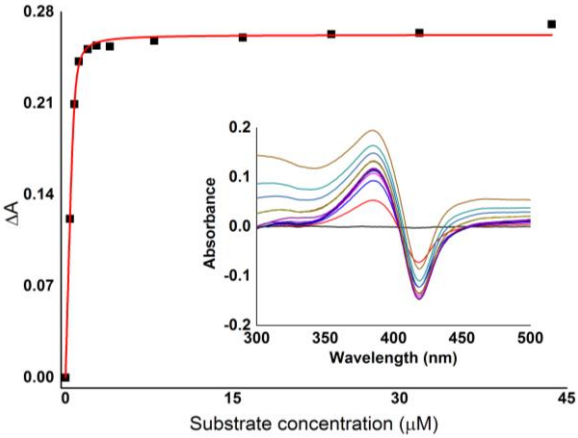
ethyl cyclohexylacetate
(1.65 μM CYP101B1/K_d 40 μM)



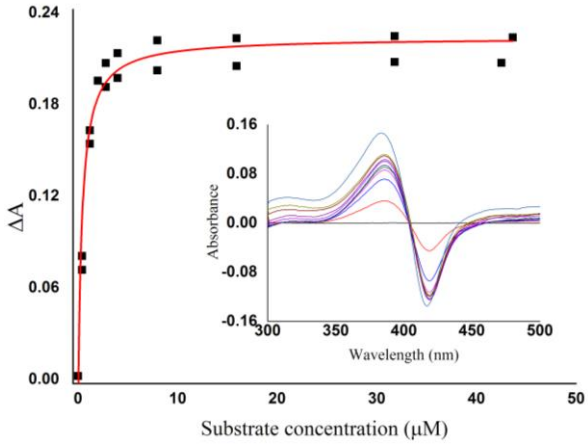
cyclooctyl acetate
(1.6 μM CYP101B1/K_d 1.6 μM)



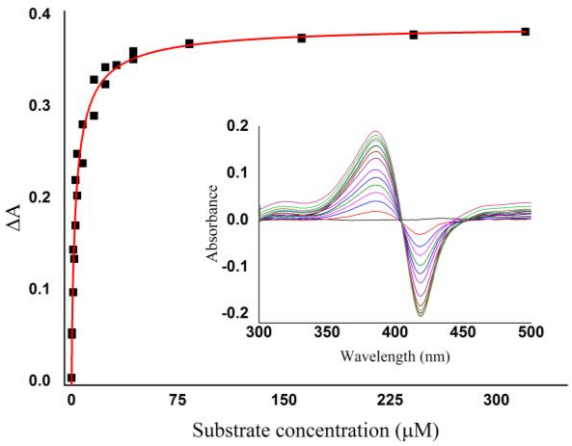
cyclooctyl isobutyrate
(1.5 μM CYP101B1/K_d 1.3 μM)



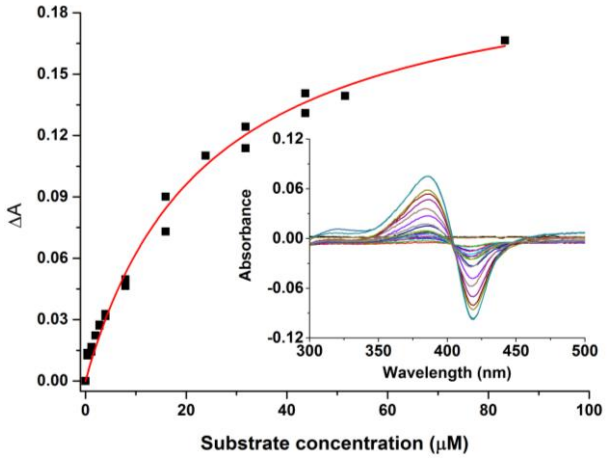
cyclododecyl acetate
(2.1 μM CYP101B1/K_d 0.05 μM)



cyclododecane
(2.37 μM CYP101B1/K_d 0.16 μM)



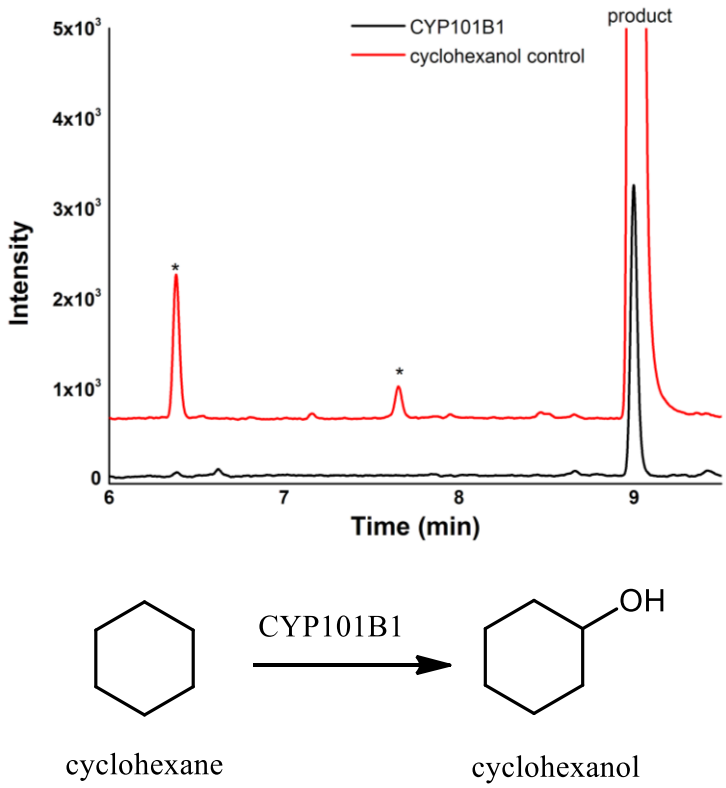
cyclododecanol
(3.15 μM CYP101B1/K_d 1.5 μM)



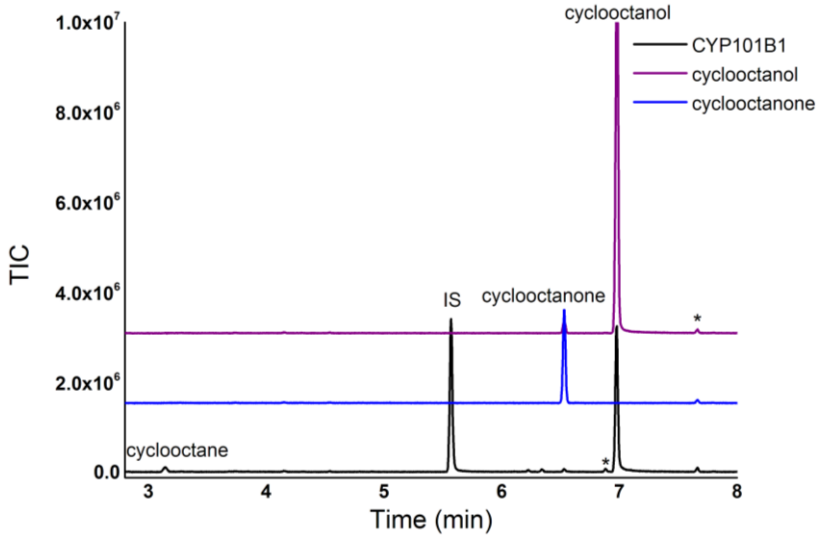
cyclooctane
(2.19 μM CYP101B1 /K_d 31 μM)

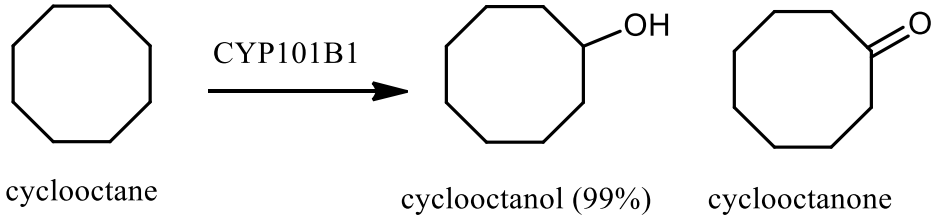
Figure S3 The GC-FID or GC-MS analysis of the CYP101B1 catalysed turnovers of the substrates tested in this work.

(a) GC analysis of cyclohexane turnover (black). Cyclohexane (RT 2.05 min, not shown) and the product; cyclohexanol (8.9 min, control in red). Impurities are labelled (*).



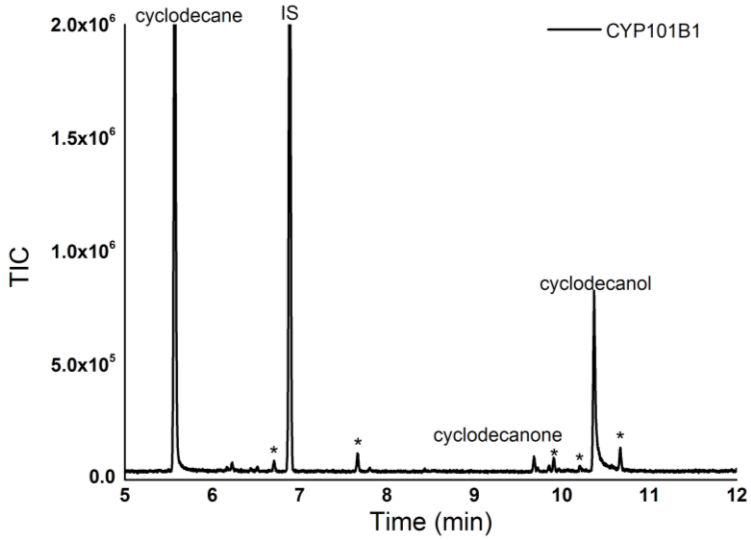
(b) The GC-MS analysis of cyclooctane turnover by CYP101B1 (black). Cyclooctane (RT 3.2 min) and the products; cyclooctanone (6.6 min, control in blue) and cyclooctanol (6.9 min, control in purple). Impurities are labelled (*).



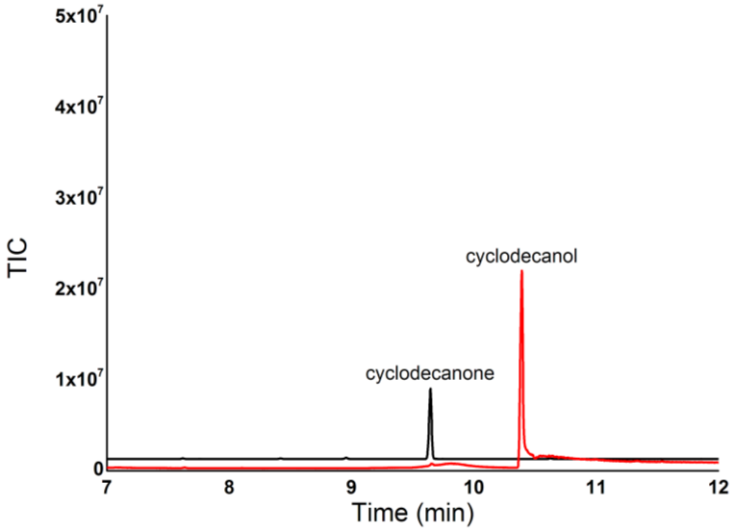


cyclooctane cyclooctanol (99%) cyclooctanone

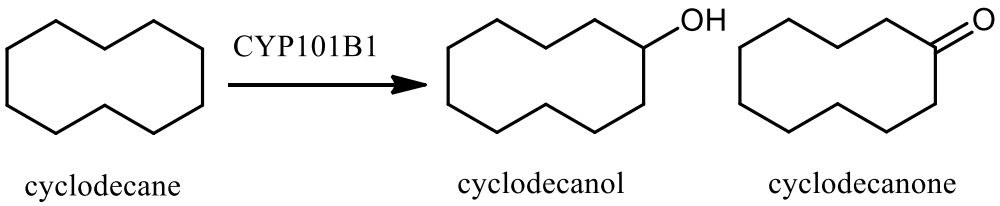
(c) The GC-MS analysis of the turnover of (i) cyclodecane by CYP101B1 (black). Cyclodecane (RT 5.1 min) and the products; cyclodecanone (9.7 min) and cyclodecanol (10.45 min). (ii) The controls of cyclodecanone and cyclodecanol. Cyclodecanol was synthesised from the reduction of cyclodecanone with sodium borohydride ($NaBH_4$) using standard method [5]. Impurities are labelled (*). The internal standard (*p*-cresol is shown at 6.8 min).



(i)

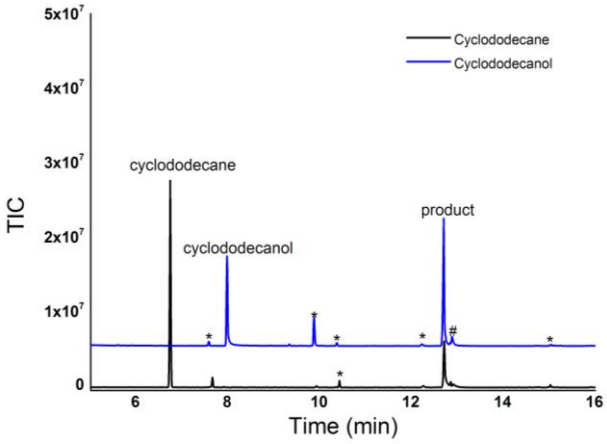


(ii)

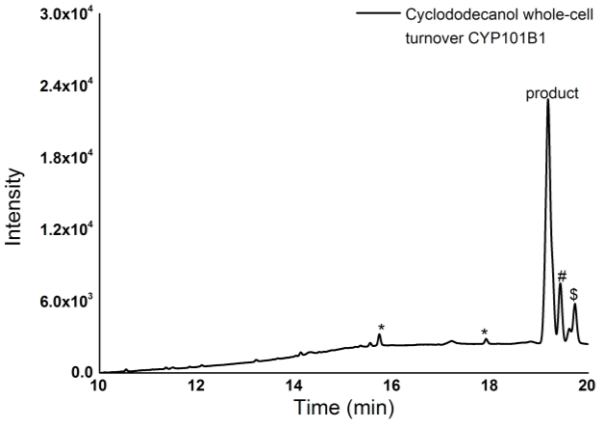


cyclodecane cyclodecanol cyclodecanone

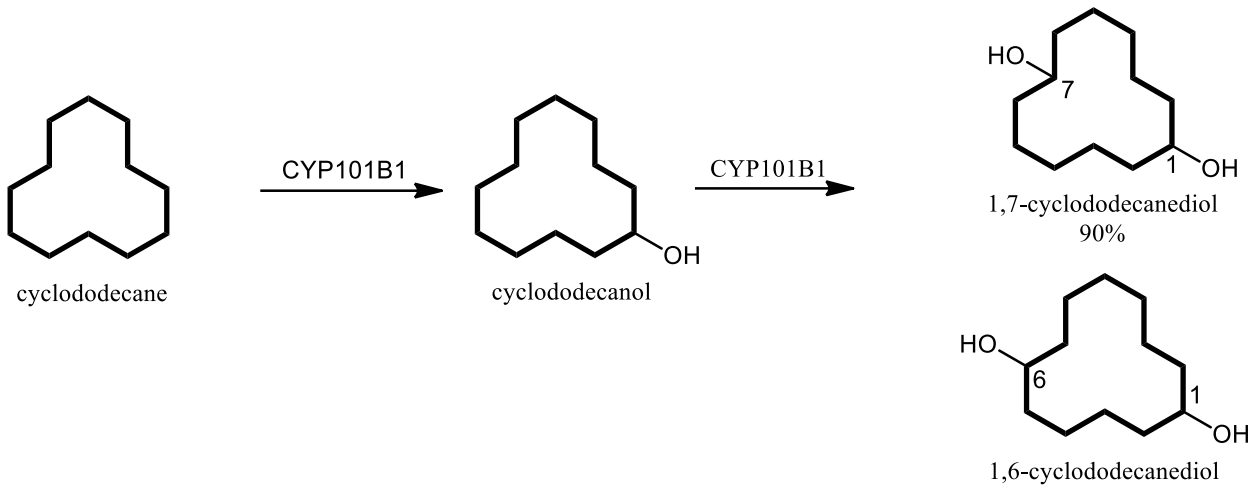
(d) (i) GC-MS analysis of the turnover of cyclododecane and cyclododecanol by CYP101B1. Cyclododecane (RT 6.8 min), cyclododecanol (RT 7.9 min) and the products; 1,7-cyclododecanediol (RT 12.9 min) and minor metabolite# 1,6-cyclododecanediol ((RT 12.95 min). (ii) The GC analysis of the whole-cell turnover of cyclododecanol. Cyclododecanol (RT 10.2 min) and the product; 1,7-cyclododecanediol (RT 19.6 min) and minor metabolite 1,6-cyclododecanediol (RT 19.7 min) and a minor unidentified product which was tentatively assigned as either 1,4-cyclododecanediol or the diastereomer of 1,6-cyclododecanediol (RT 19.8 min) based on 6 equal intensity peaks in the ^{13}C NMR and its MS (See Figure S5ii and Table S1). Impurities are labelled (*).



(i)



(ii)



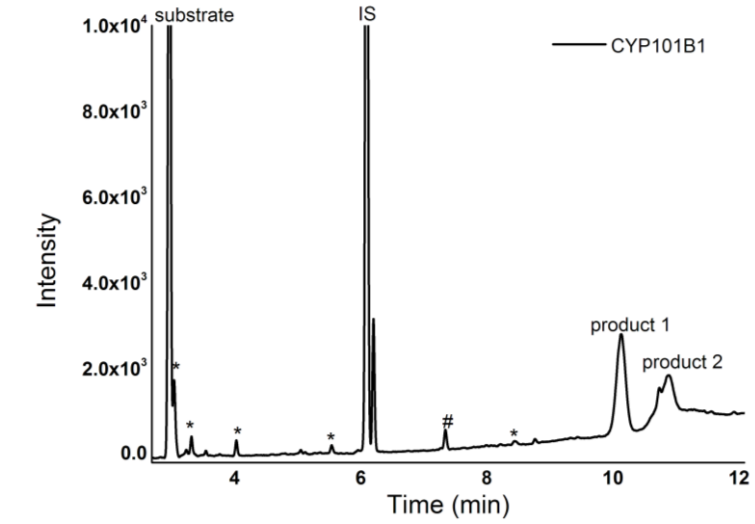
cyclododecane

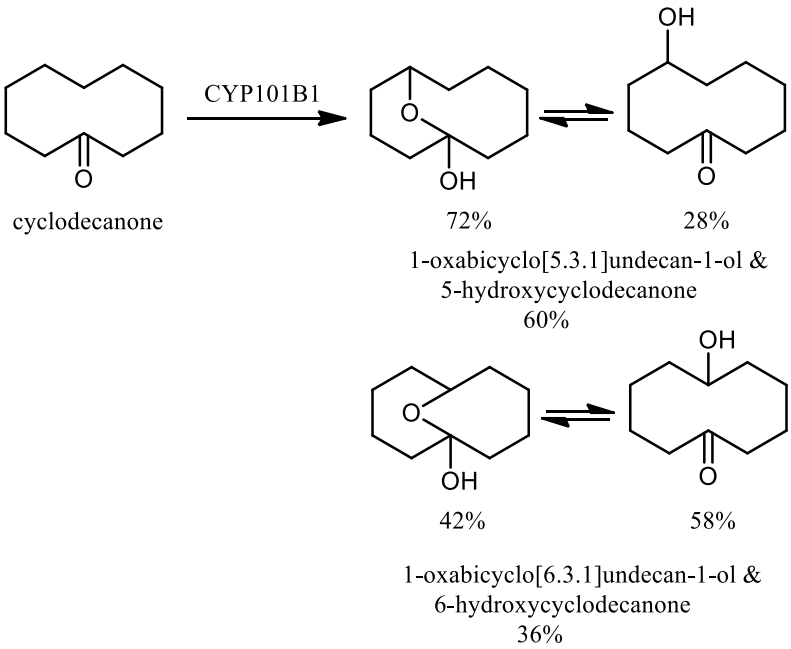
cyclododecanol

1,7-cyclododecanediol
90%

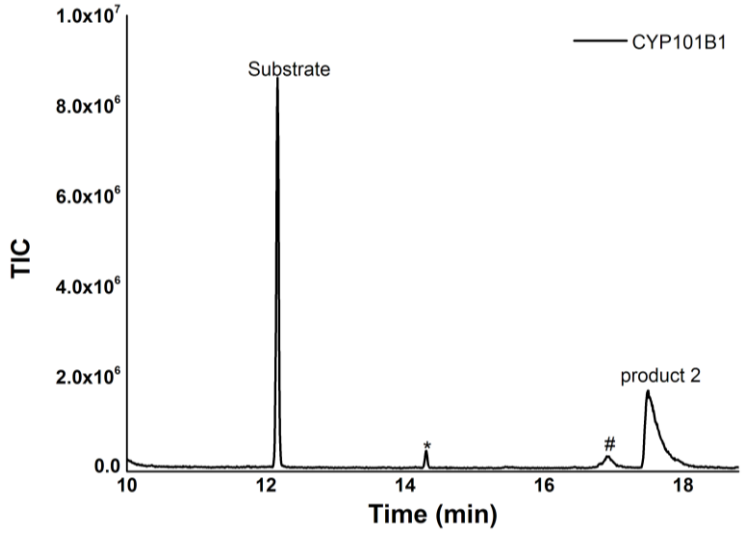
1,6-cyclododecanediol

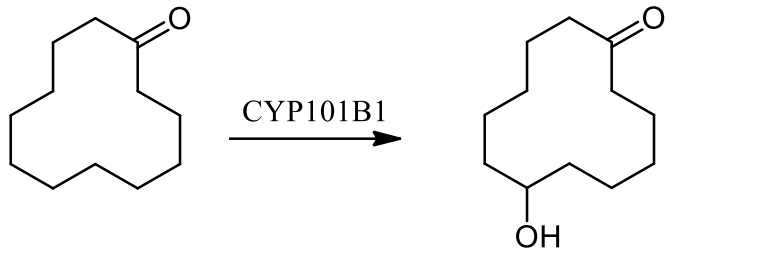
(f) The GC-FID analysis of the *in vitro* turnover of cyclodecanone by CYP101B1. Cyclodecanone (RT 2.9 min) and the products; 1-oxabicyclo[5.3.1]undecan-1-ol in a mixture with 5-hydroxycyclodecanone (RT 10.0 min) and a mixture of 1-oxabicyclo[6.3.1]undecan-1-ol and 6-hydroxycyclodecanone (RT 10.9 min). Impurities are labelled (*). A potential minor product is labelled # (RT 7.2 min).





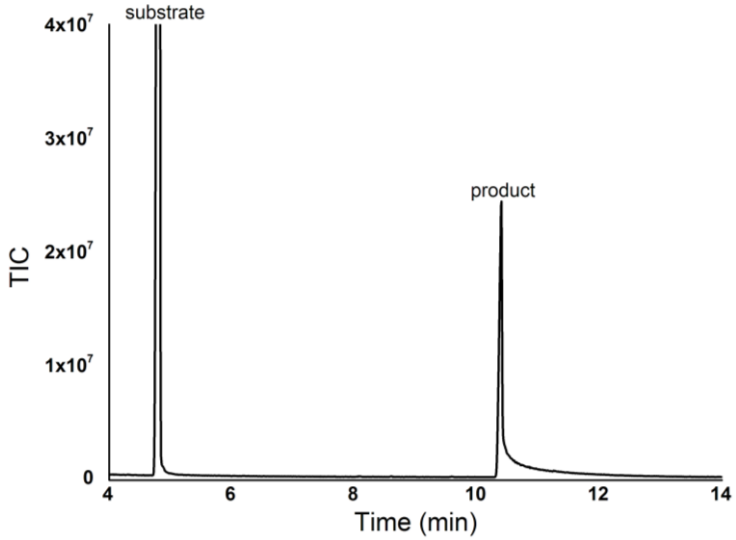
(g) The GC-MS analyses of the *in vitro* turnovers of cyclododecanone by CYP101B1. Cyclododecanone (RT 12.1 min) and the products; 7-hydroxycyclododecanone (RT 17.7 min). There is a minor product[#] in CYP101B1 enzyme turnover at RT 16.9 min the mass spectrum suggested that this was consistent with a monooxygenase product with a mass m^+/z 198.35. Impurities are labelled (*).

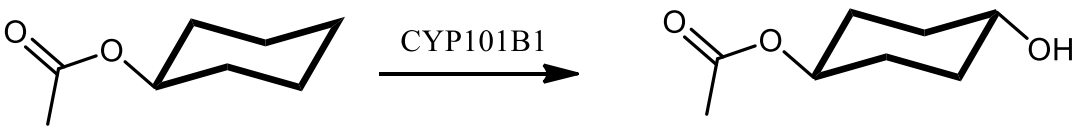




cyclododecanone 7-hydroxycyclododecanone
96%

(h) The GC-MS analysis of the *in vitro* turnover of cyclohexyl acetate by CYP101B1. Cyclohexyl acetate (RT 4.9 min) and the product; *trans*-4-hydroxycyclohexyl acetate (RT 10.6 min).

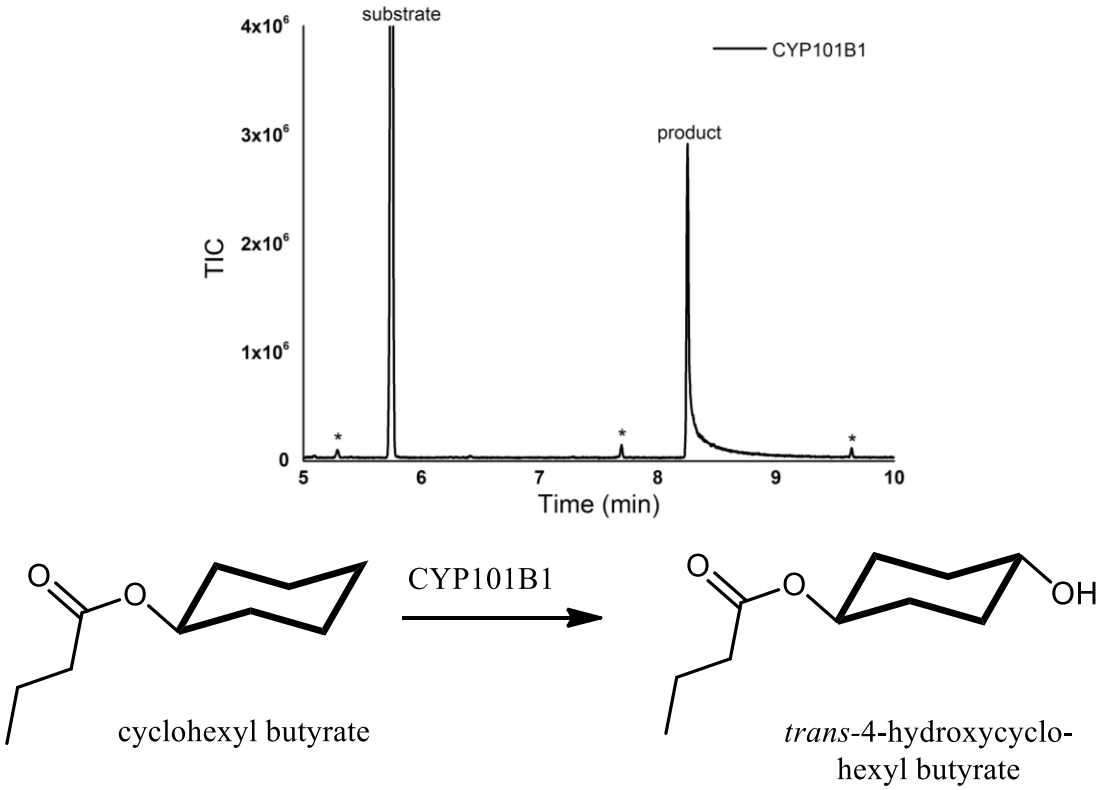




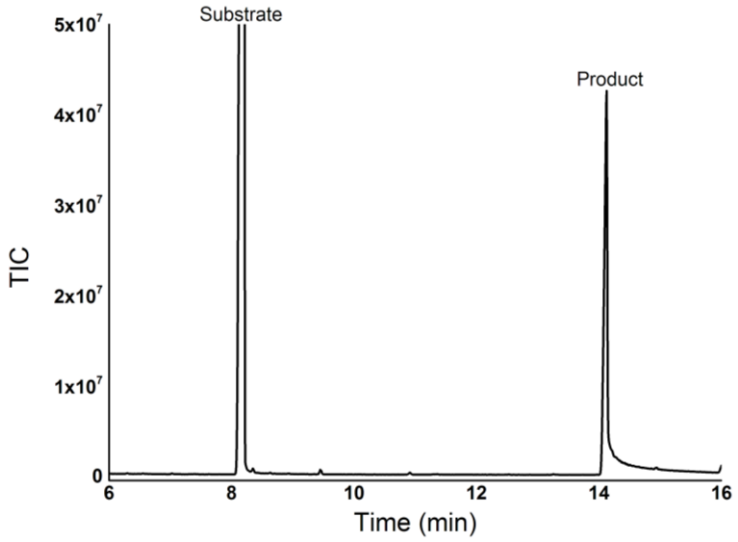
cyclohexyl acetate

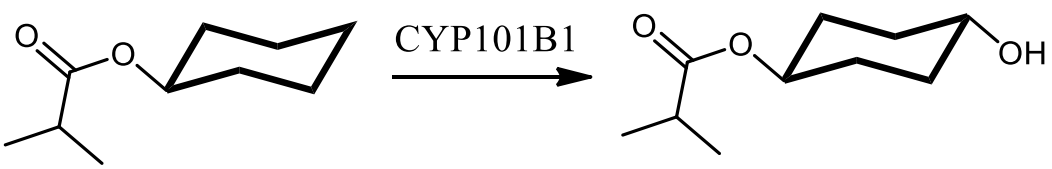
trans-4-hydroxycyclohexyl acetate

(i) The GC-MS analysis of the *in vitro* turnover of cyclohexyl butyrate by CYP101B1 (black). Cyclohexyl butyrate (RT 5.7 min) and the product; *trans*-4-hydroxy cyclohexyl butyrate (RT 8.5 min). Impurities are labelled (*).



(j) The GC-MS analysis of the *in vitro* turnover of cyclohexyl isobutyrate by CYP101B1. Cyclohexyl isobutyrate (RT 8.1 min) and the product; 4-hydroxycyclohexyl isobutyrate (RT 14.1 min).

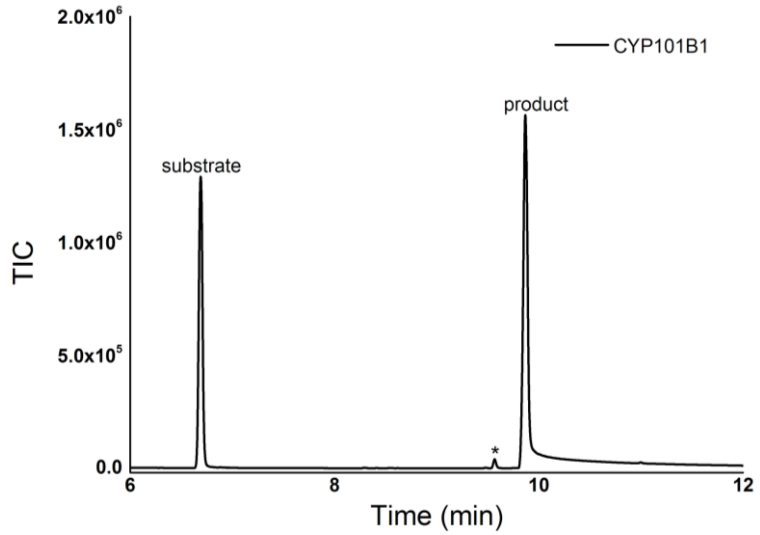


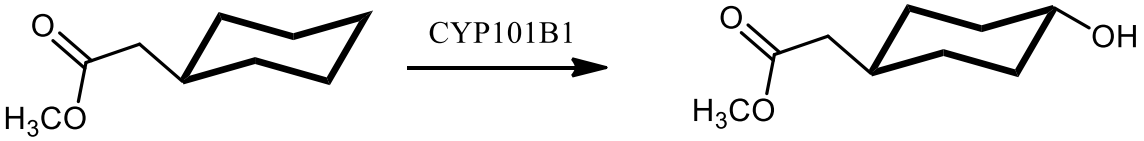


cyclohexyl isobutyrate

trans-4-hydroxycyclo-hexyl isobutyrate

(k) GC analysis of the *in vivo* turnover of methyl cyclohexylacetate by CYP101B1. Methyl cyclohexylacetate (RT 6.7 min) and the product; methyl (*trans*-4-hydroxycyclohexyl)acetate (RT 9.9 min). Impurities are labelled (*).

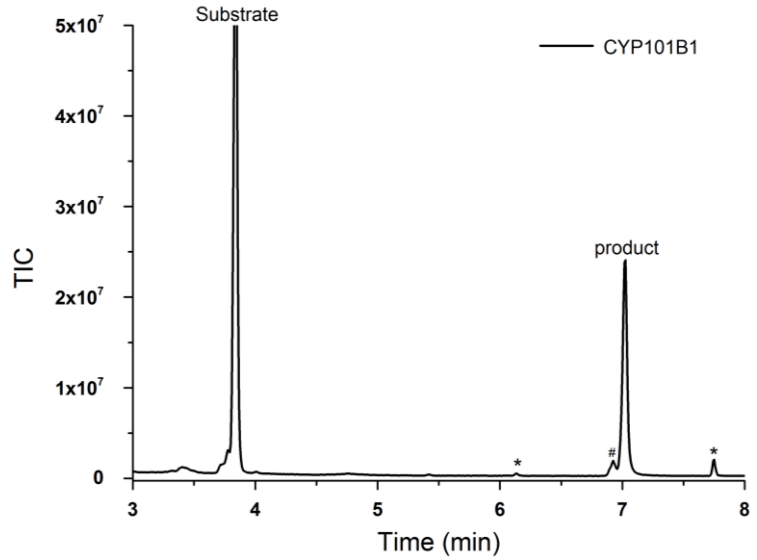


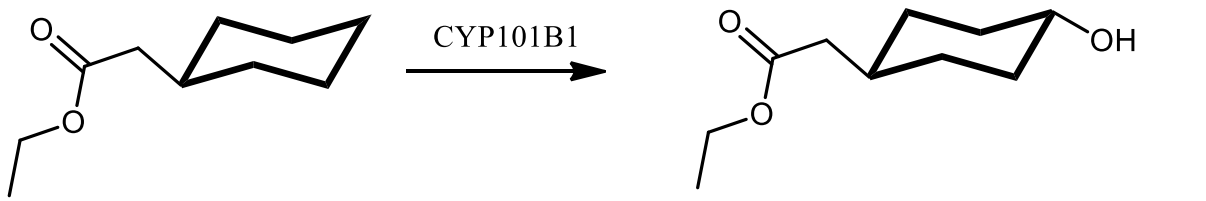


methyl cyclohexylacetate

methyl (*trans*-4-hydroxycyclohexyl)acetate

(l) GC-MS analysis of the *in vitro* turnover of ethyl cyclohexylacetate by CYP101B1. Ethyl cyclohexylacetate (RT 3.8 min) and the product; ethyl (*trans*-4-hydroxycyclohexyl)acetate (RT 7.1 min). A further oxidation metabolite (#) is highlighted at RT 6.9 min. Impurities are labelled (*).

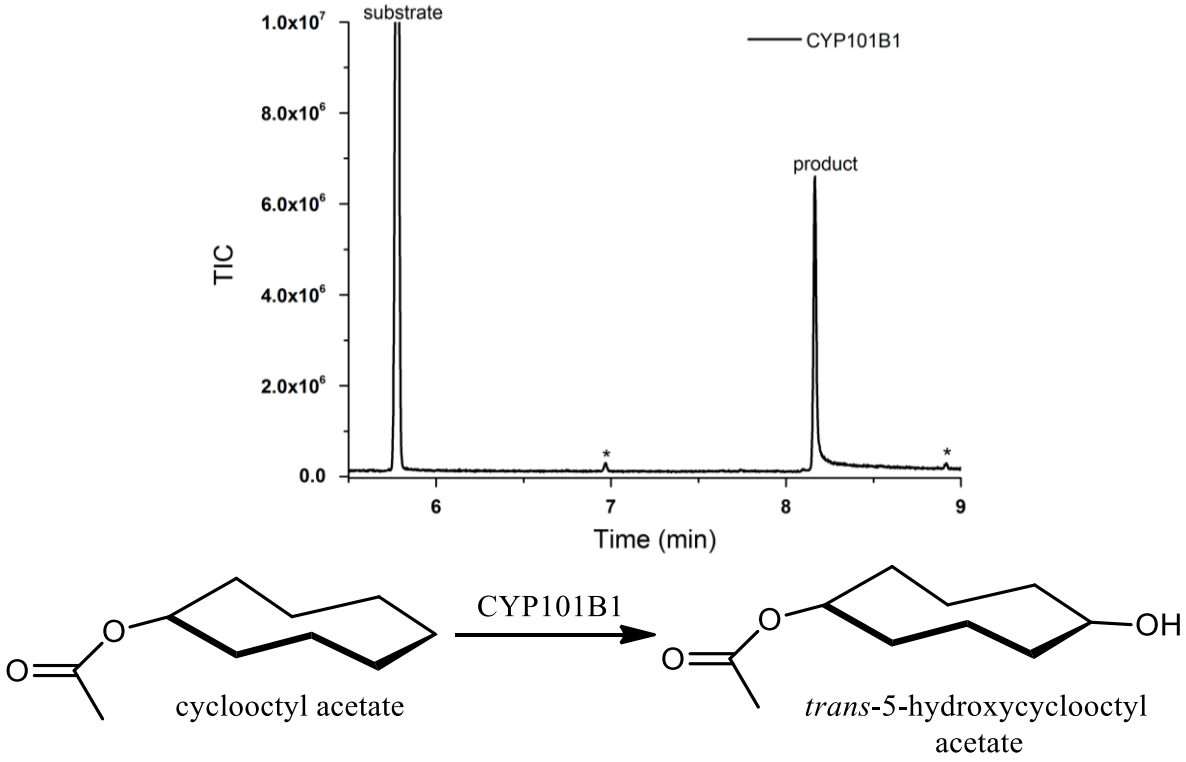




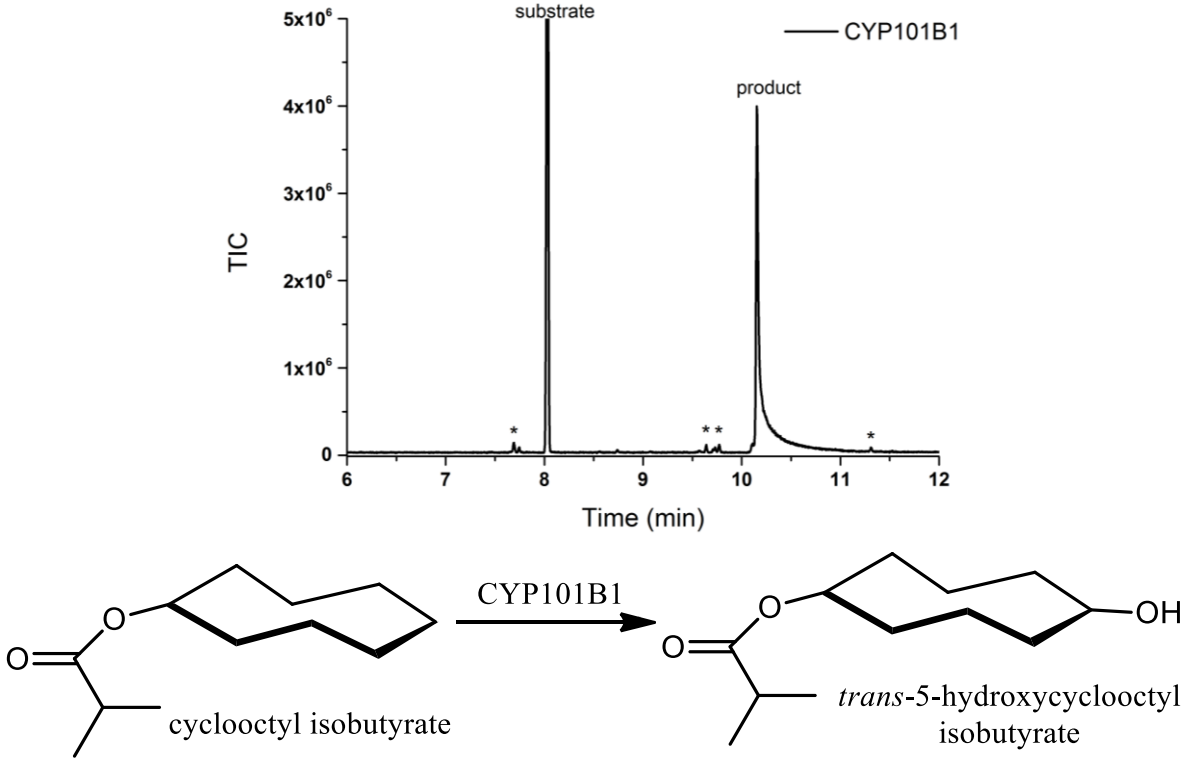
ethyl cyclohexylacetate

ethyl (*trans*-4-hydroxycyclohexyl)acetate

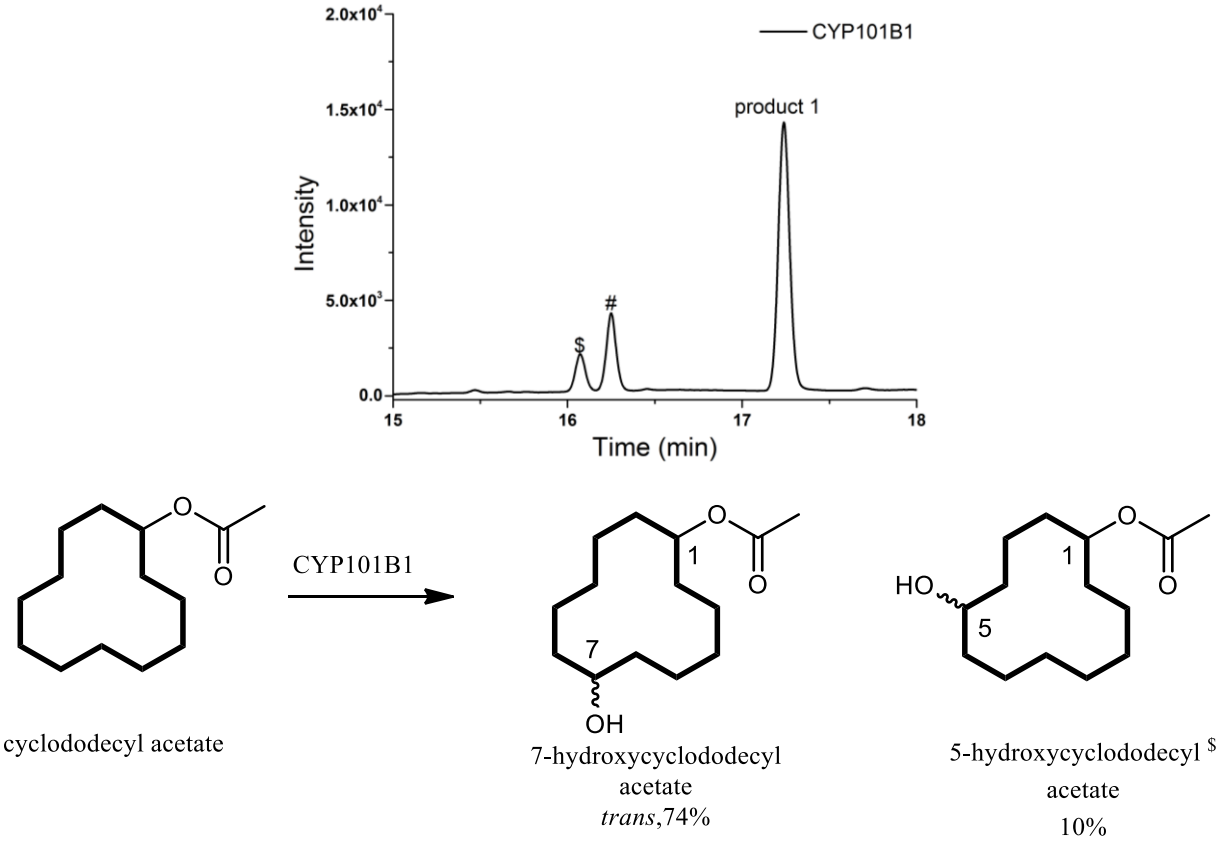
(m) GC-MS analysis of the *in vitro* turnover of cyclooctyl acetate by CYP101B1. Cyclooctyl acetate (RT 5.6 min) and the product; *trans*-5-hydroxycyclooctyl acetate (RT 8.2 min). Impurities are labelled (*).



(n) GC-MS analysis of the *in vitro* turnover of cyclooctyl isobutyrate by CYP101B1. Cyclooctyl isobutyrate (RT 8.0 min) and the product; *trans*-5-hydroxycyclooctyl isobutyrate (RT 10.2 min). Impurities are labelled (*).



(o) The GC (left) analyses of *in vitro* turnover of cyclododecyl acetate by CYP101B1. Cyclododecyl acetate (RT 5.6; not shown) and the products; 5-hydroxycyclododecyl acetate[$] (RT 16.2, *trans*), an unidentified hydroxyl-metabolite[#] (RT 16.4), 7-hydroxycyclododecyl acetate (product 1, RT 17.3; *trans*) [6]. Another unknown minor products (≤ 2%) is present in the NMR analysis (Figure S5). Impurities are labelled (*).



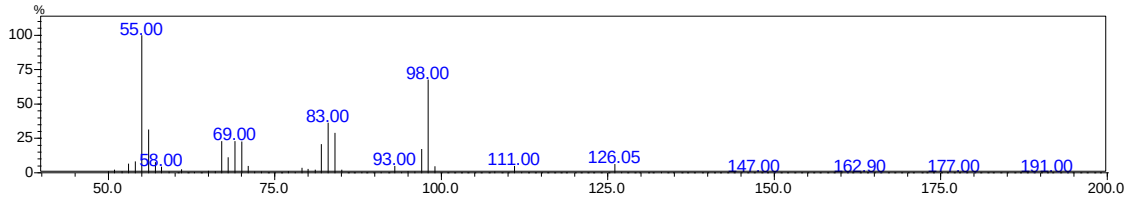
5-Hydroxycyclododecyl acetate was confirmed by coeluting the NMR sample in GC. The product at 16.4 min was another alcohol potentially the other diastereomer of 5-hydroxycyclododecyl acetate (Figure S5).

The final product distribution was assigned as follows; 7-hydroxycyclododecyl acetate 74% (*trans*) with approx. 2% of the *cis* isomer [$]5-hydroxycyclododecyl acetate 10% (*trans* isomer). The remaining unknown product (14%) is either an unknown hydroxylated product or other diastereomer of 5-hydroxy product.

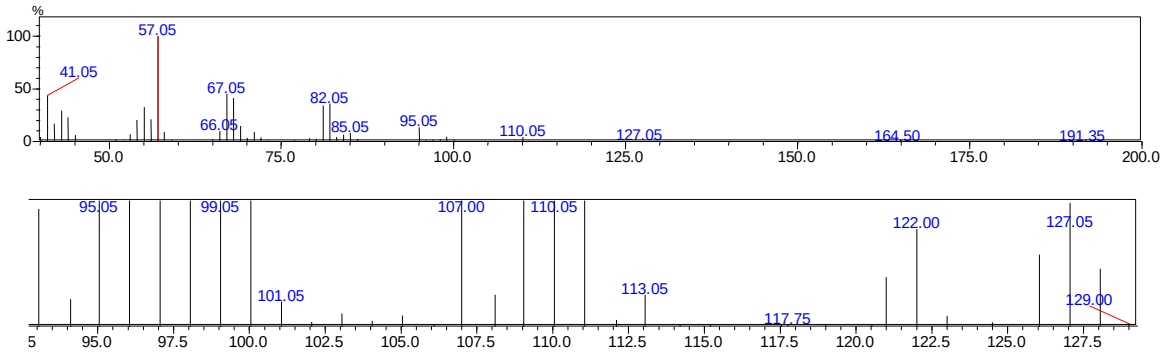
Figure S4 Mass spectra data

MS analysis of cyclooctane products

MS analysis of cyclooctanone RT 6.6 min (m^+/z = 126.05)



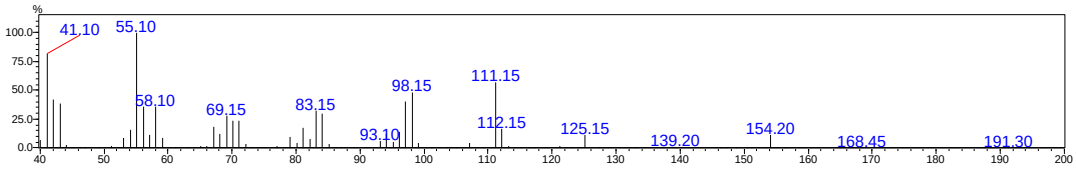
MS analysis of cyclooctanol RT 6.9 min (m^+/z = 128.05)



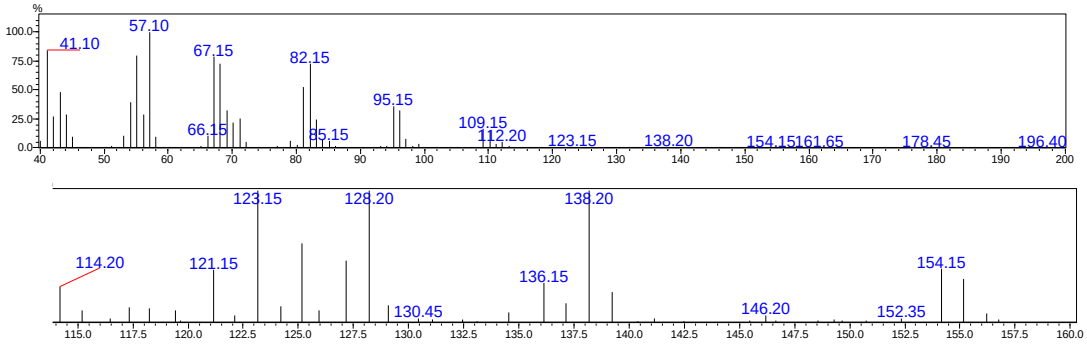
Expanded version

MS analysis of cyclodecane products

MS analysis of cyclodecanone RT 9.7 min (m^+/z = 154.20)



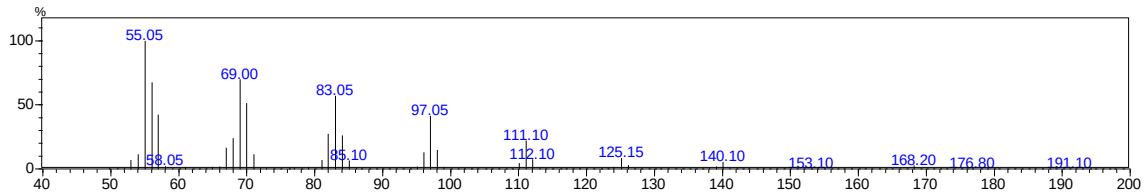
MS analysis of cyclodecanol RT 10.45 min (m^+/z = 156.20)



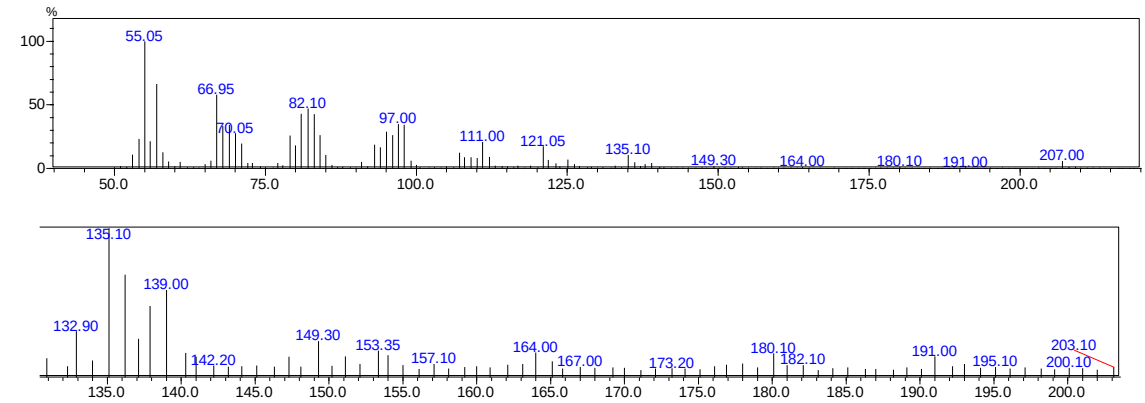
Expanded version

MS analysis of cyclododecane and cyclododecanol products

MS analysis of cyclododecane RT 6.8 min (m^+/z = 168.20)

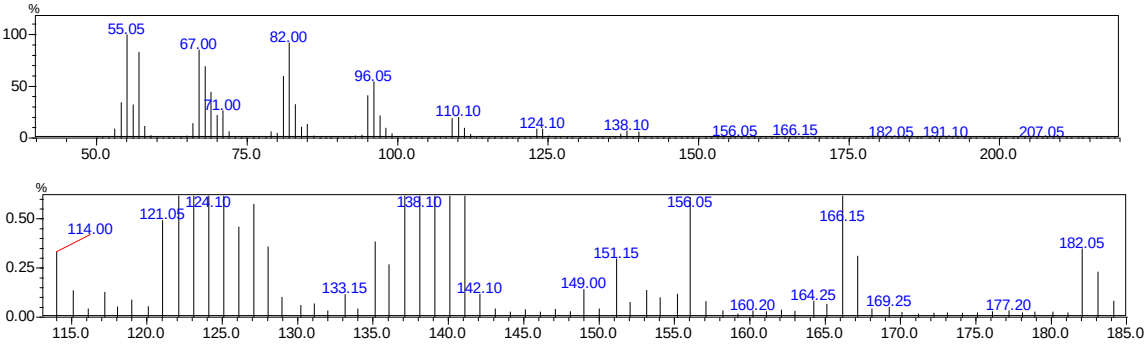


MS analysis of 1,7-cyclododecanediol RT 12.9 min (m^+/z = 200.10)



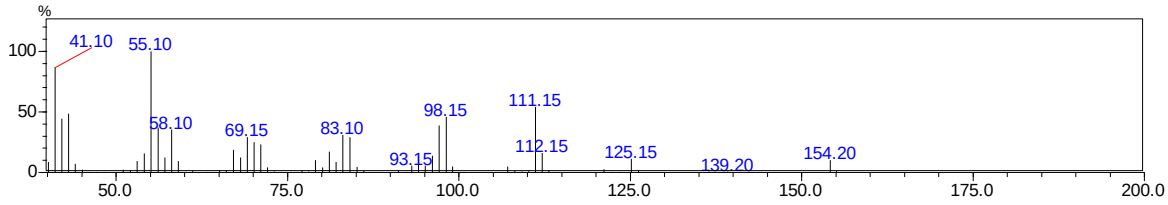
Expanded version

MS analysis of cyclododecanol control RT 7.9 min (m^+/z = 184.2)



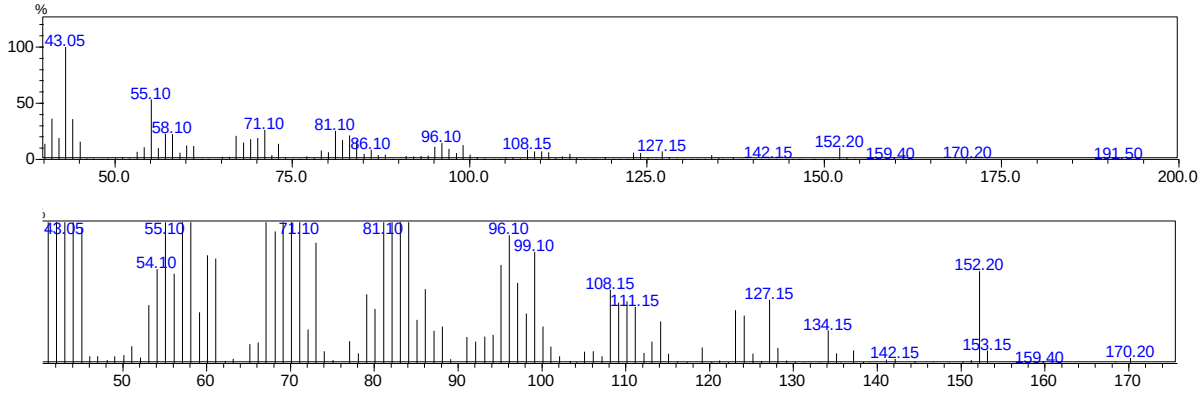
MS analysis of cyclodecanone products

MS analysis of cyclodecanone RT 7.5 min (m^+/z =154.20)



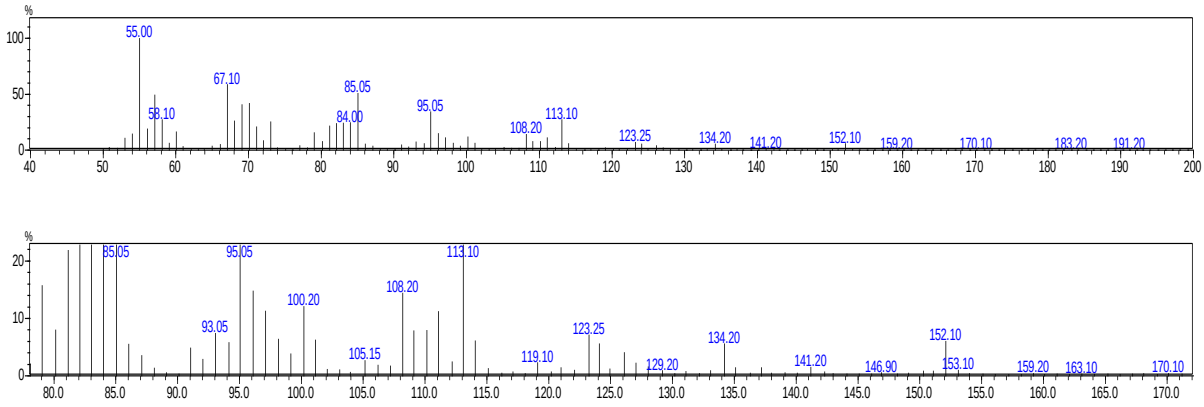
MS analysis of 1-oxabicyclo[5.3.1]undecan-1-ol and 5-hydroxycyclodecanone mixture RT 10.1 min (m^+/z = 170.10) CYP101B1 [7]

In vitro turnover product



Expanded version

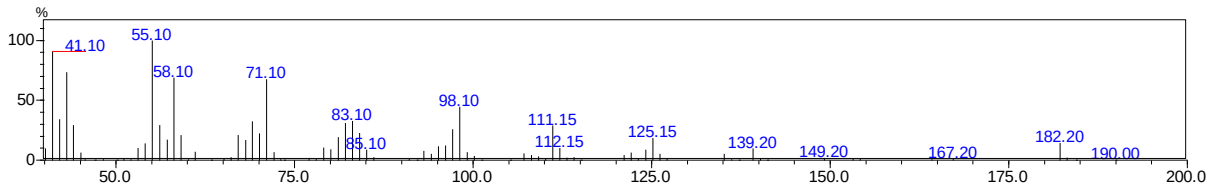
MS analysis of 1-oxabicyclo[6.3.1]undecan-1-ol and 6-hydroxycyclodecanone RT 10.25 min (m^+/z = 170.1) CYP101B1



Expanded version

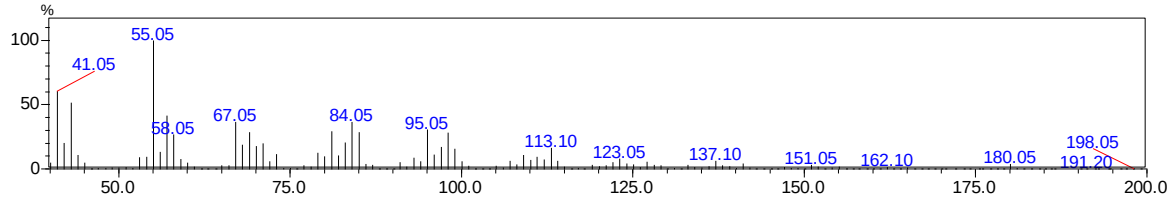
MS analysis of cyclododecanone products

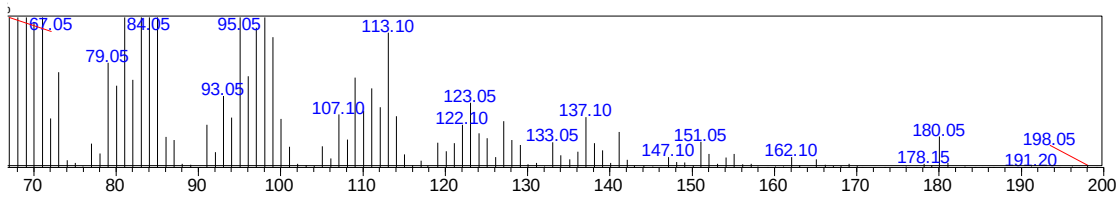
MS analysis of cyclododecanone RT 12.1 min (m^+/z = 182.2)



MS analysis of 7-hydroxycyclododecanone RT 17.7 min (m^+/z = 198.50) [8]

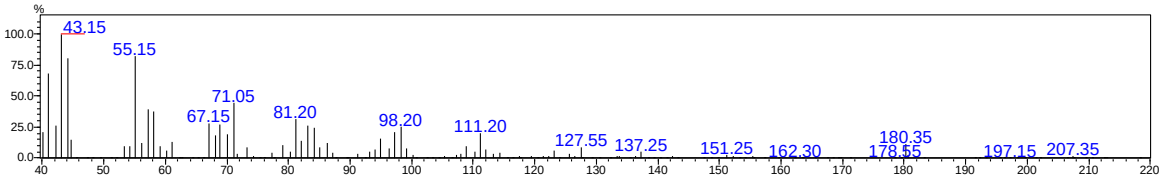
Experimental m^+/z = 198.05, 180.05, 162.10, 151.05, 137.1, 123.05, 113.1, 95.05, 84.05, 84.05, 81.10, 67.05. 55.00 and 41.10
Reported m^+/z = 198, 180, 109, 81 and 55

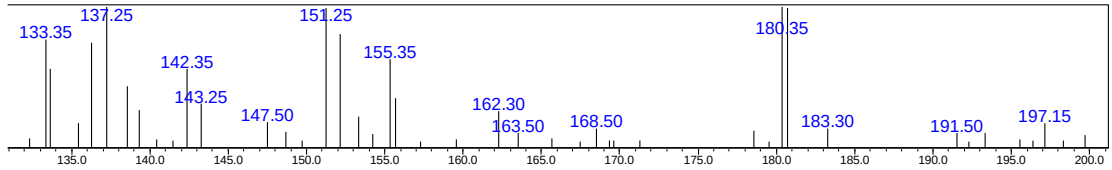




Expanded version

MS analysis of unidentified metabolite RT 16.9 min (m^+/z = 198.35)
Experimental m^+/z = 198.35, 180.35, 162.30, 155.35, 151.25, 137.25, 127.55, 111.20, 98.20, 81.20, 71.05, 67.15, 55.15 and 43.15

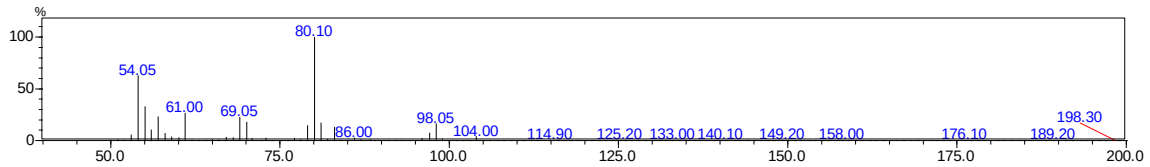




Expanded version

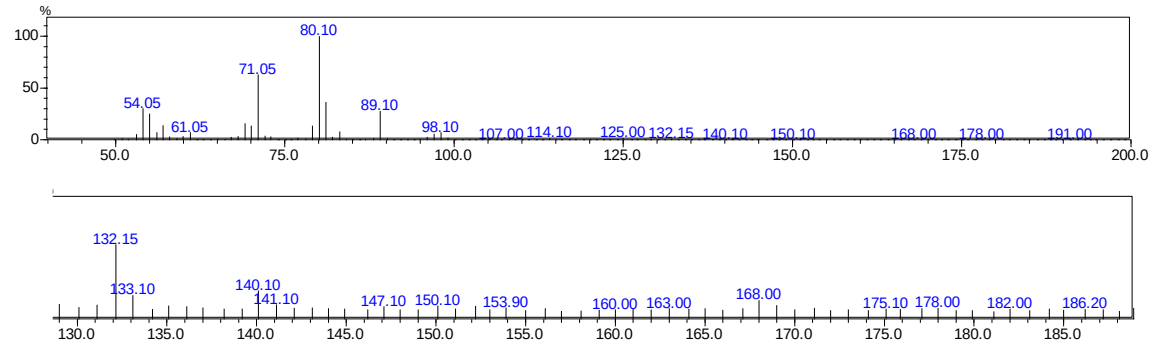
MS analysis of cyclohexyl acetate product

MS analysis of *trans*-4-hydroxycyclohexyl acetate RT 10.6 min (m^+/z = 158.00)



MS analysis of cyclohexyl butyrate product

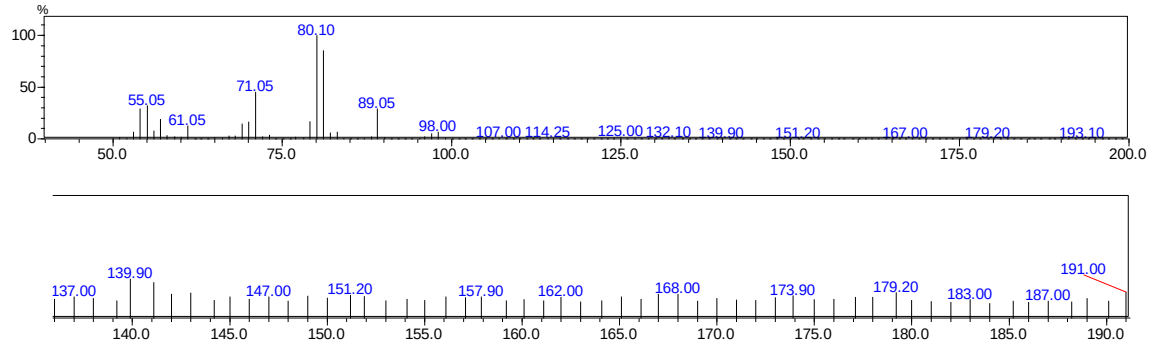
MS analysis of *trans*-4-hydroxycyclohexyl butyrate RT 8.5 min (m^+/z = 186.20)



Expanded version

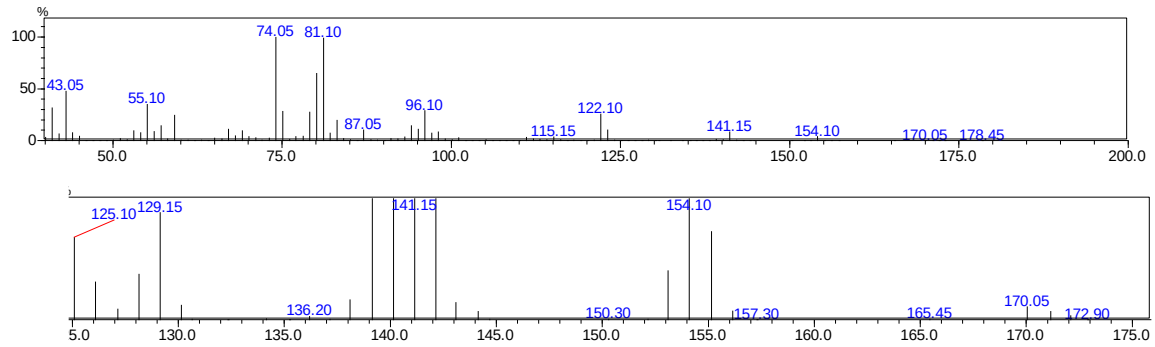
MS analysis of cyclohexyl isobutyrate product

MS analysis of 4-hydroxycyclohexyl isobutyrate RT 14.1 min (m^+/z = 186.0)



MS analysis of methyl cyclohexylacetate product

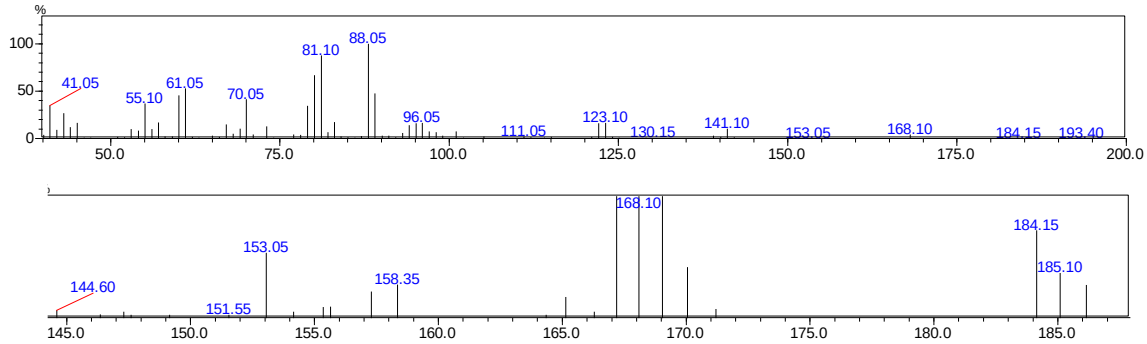
MS analysis of methyl (*trans*-4-hydroxycyclohexyl)acetate RT 9.9 min (m^+/z = 172.0)



Expanded version

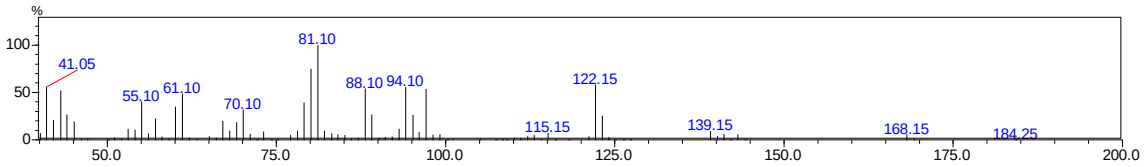
MS analysis of ethyl cyclohexylacetate

MS analysis of ethyl (*trans*-4-hydroxycyclohexyl)acetate RT 7.0 min (m^+/z = 186.15)



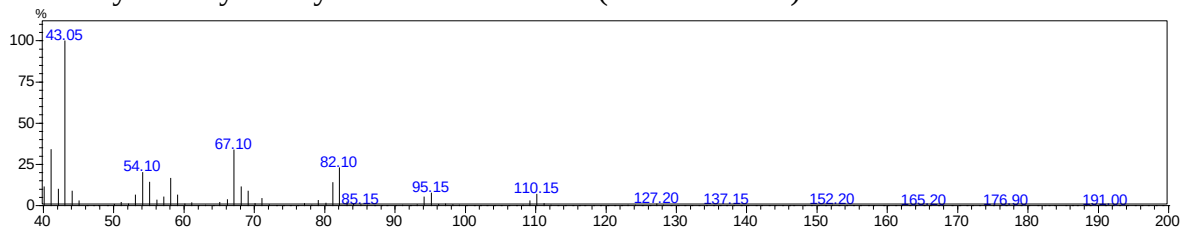
Expanded version

MS analysis of further oxidation product of ethyl (*trans*-4-hydroxycyclohexyl)acetate RT 6.9 min (m^+/z = 184.25)

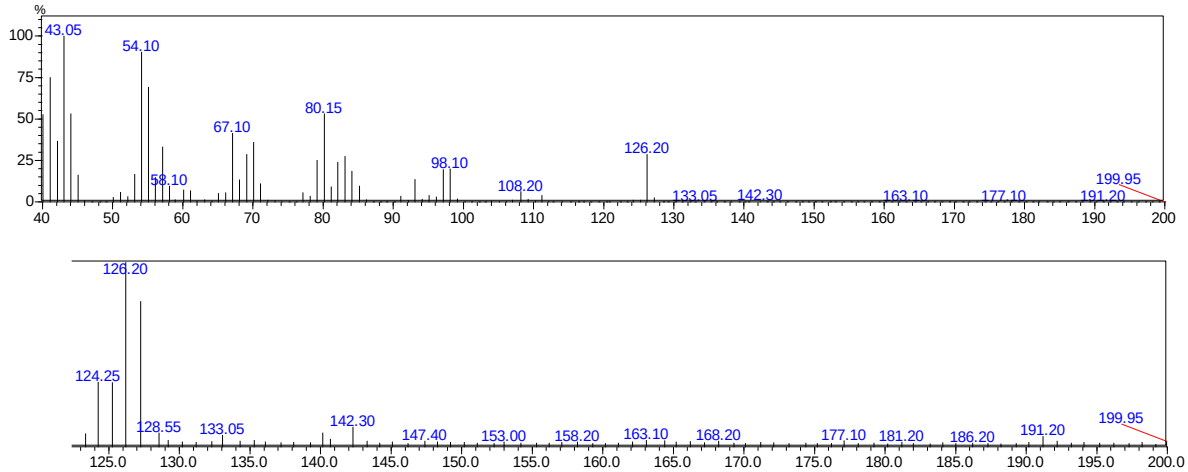


MS analysis of cyclooctyl acetate products

MS analysis of cyclooctyl acetate RT 5.6 min (m^+/z = 170.10)



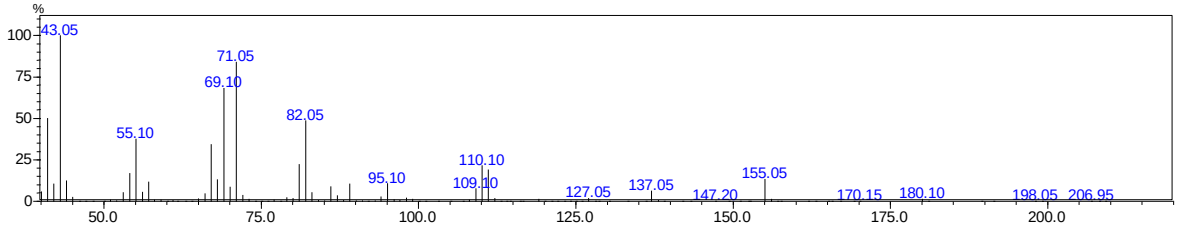
MS analysis of *trans*-5-hydroxycyclooctyl acetate RT 8.2 min (m^+/z = 186.20)



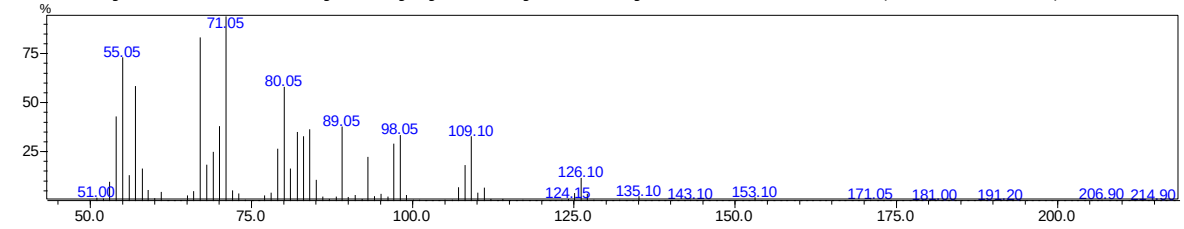
Expanded version

MS analysis of cyclooctyl isobutyrate products

MS analysis of cyclooctyl isobutyrate RT 8.0 min (m^+/z = 198.05)

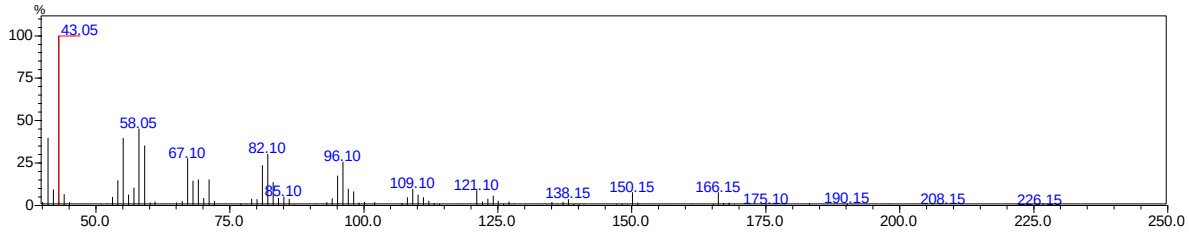


MS analysis of *trans*-5-hydroxycyclooctyl isobutyrate RT 10.2 min (m^+/z = 214.9)

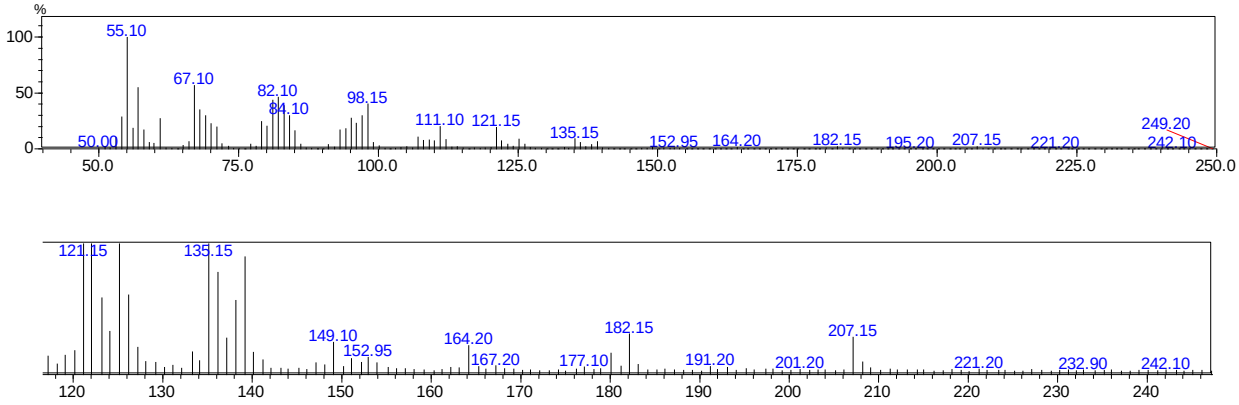


MS analysis of cyclododecyl acetate products

MS analysis of cyclododecyl acetate RT 9.6 min (m^+/z = 226.15)



MS analysis of 7-hydroxycyclododecyl acetate (*trans*) RT 13.6 min (m^+/z = 242.10)



Expanded version

MS analysis of 5-hydroxycyclododecyl acetate RT 12.4 min

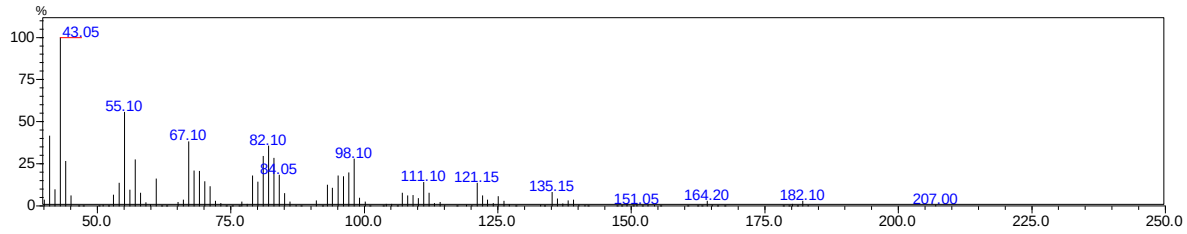


Figure S5 NMR analysis of the metabolites generated by CYP101B1

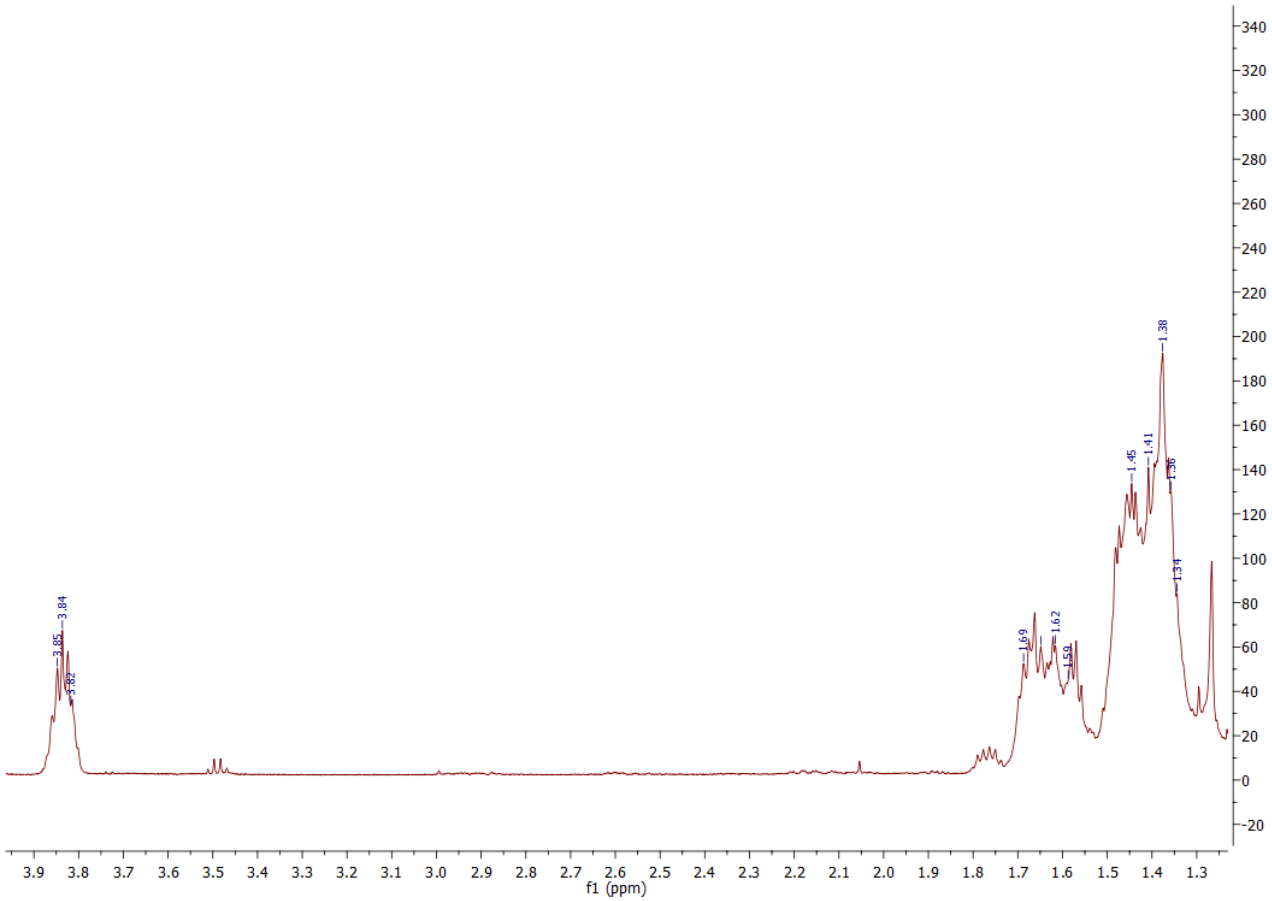


Figure S5 i ^{1}H NMR of the mixture of alcohols isolated after the CYP101B1 catalysed oxidation of cyclododecanol (1,7-cyclododecanediol, 1,6-cyclododecanediol and a third minor product). The metabolites were assigned using the carbon signals and their intensities (see Table S1).

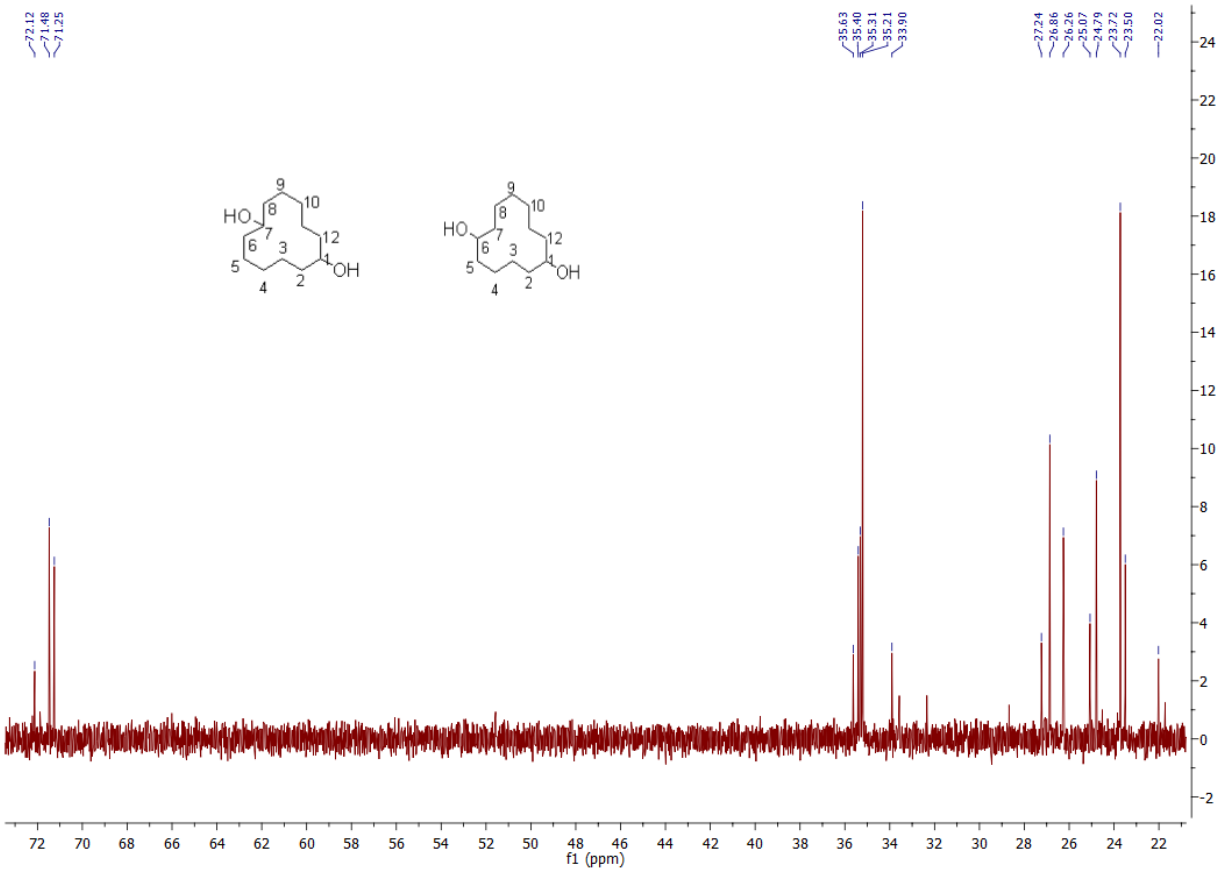


Figure S5 ii ^{13}C NMR of the mixture of alcohols isolated after the CYP101B1 catalysed oxidation of cyclododecanol (1,7-cyclododecanediol, 1,6-cyclododecanediol and a third minor product). The details of the carbon signals and their intensities are given in Table S1 (below).

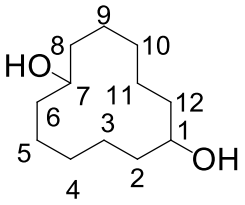
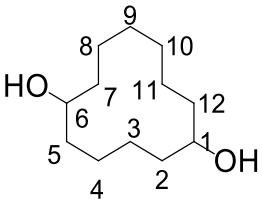
		1,7-cyclododecanediol [9] (4 signals) 2:4:2:4 intensity	1,6-cyclododecanediol (6 signals) equal intensity	Remaining (6 signals) equal intensity
Atom	**Intensity**			potentially a diastereomer of C6 or C4 hydroxy?
72.12	1			
71.48	2	C1, C7		
71.25	2		C1, C6	
35.63	1			
35.40	2		C2, C5	
35.31	2		C7, C12	
35.21	4	C2, C6, C8, C12		
33.90	1			
27.24	1			
26.86	2	C4, C10		
26.26	2		C3, C4	
25.07	1			
24.79	2		C8, C11	
23.72	4	C3, C5, C9, C11		
23.5	2		C9, C10	
22.02	1			

Table S1. The assignments of cyclododecanol metabolites using ^{13}C NMR (Figure S5 ii).

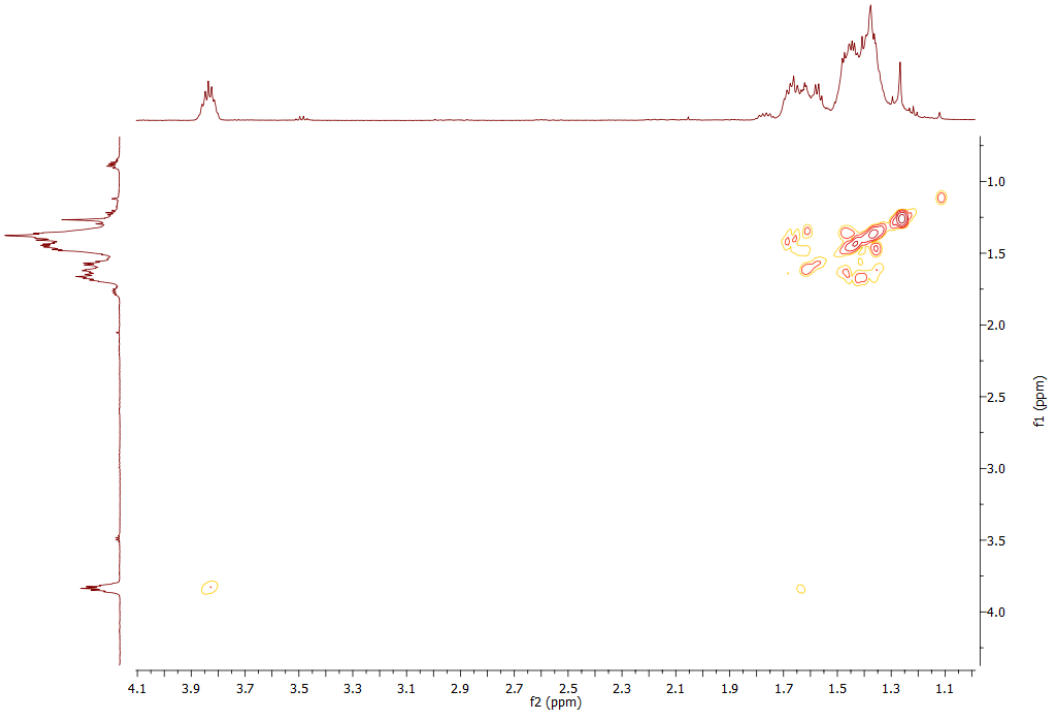


Figure S5 iii gCOSY NMR of the mixture of alcohols isolated after the CYP101B1 catalysed oxidation of cyclododecanol (1,7-cyclododecanediol, 1,6-cyclododecanediol and a third minor product).

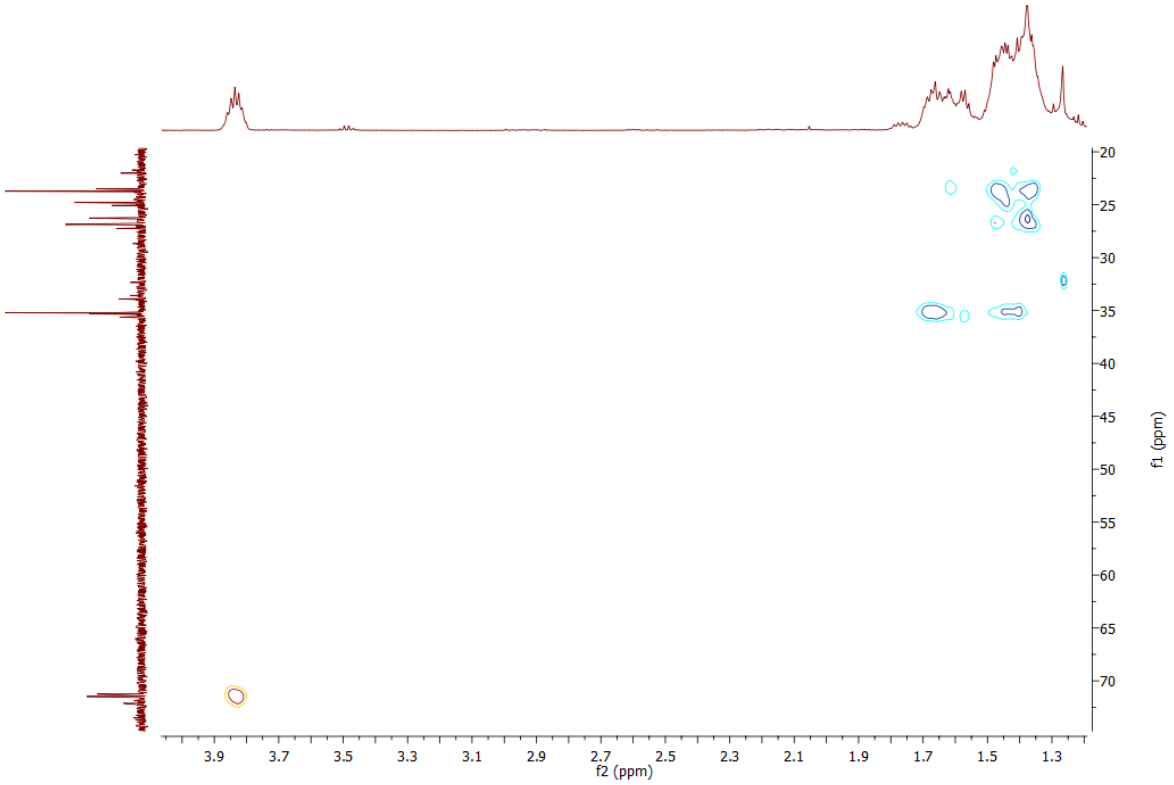


Figure S5 iv HSQC NMR of the mixture of alcohols isolated after the CYP101B1 catalysed oxidation of cyclododecanol.

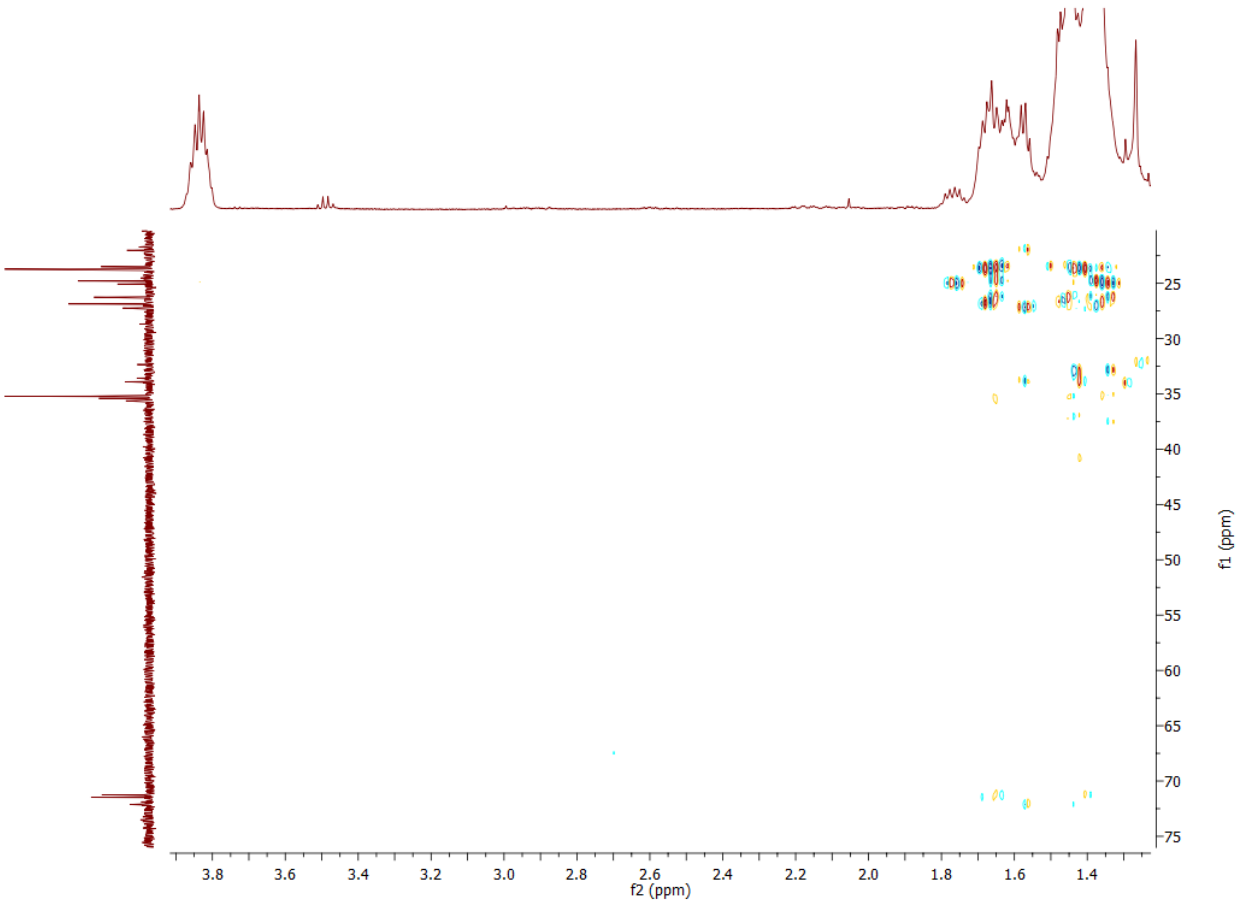


Figure S5 v Expanded HMBC NMR of the mixture of alcohols isolated after the CYP101B1 catalysed oxidation of cyclododecanol.

1-oxabicyclo[5.3.1]undecan-1-ol and 5-hydroxycyclodecanone

^{1}H NMR of 1-oxabicyclo[5.3.1]undecan-1-ol (500 MHz, $CDCl_3$) δ 4.07-4.97 (m, 1H, H5), 2.50-2.43 (m, 1H, O**H1**(C1)), 1.92-1.72 (m, 8H, H2, H3, H6, H7, H8, H9 & 2xH10), 1.71-1.61 (m, 4H, H2, H4, H7 & H8), 1.60-1.47 (m, 3H, H2, H4 & H9), 1.40-1.33 (m, 1H, H3), 1.2-1.15 (m, 1H, H6).

^{13}C NMR (126 MHz, $CDCl_3$) δ 98.85 (C1), 76.33 (C5), 40.33 (C2), 39.95 (C10), 35.24 (C6), 34.74 (C4), 30.48 (C3), 27.70 (C9), 24.42 (C8), 19.94 (C7).

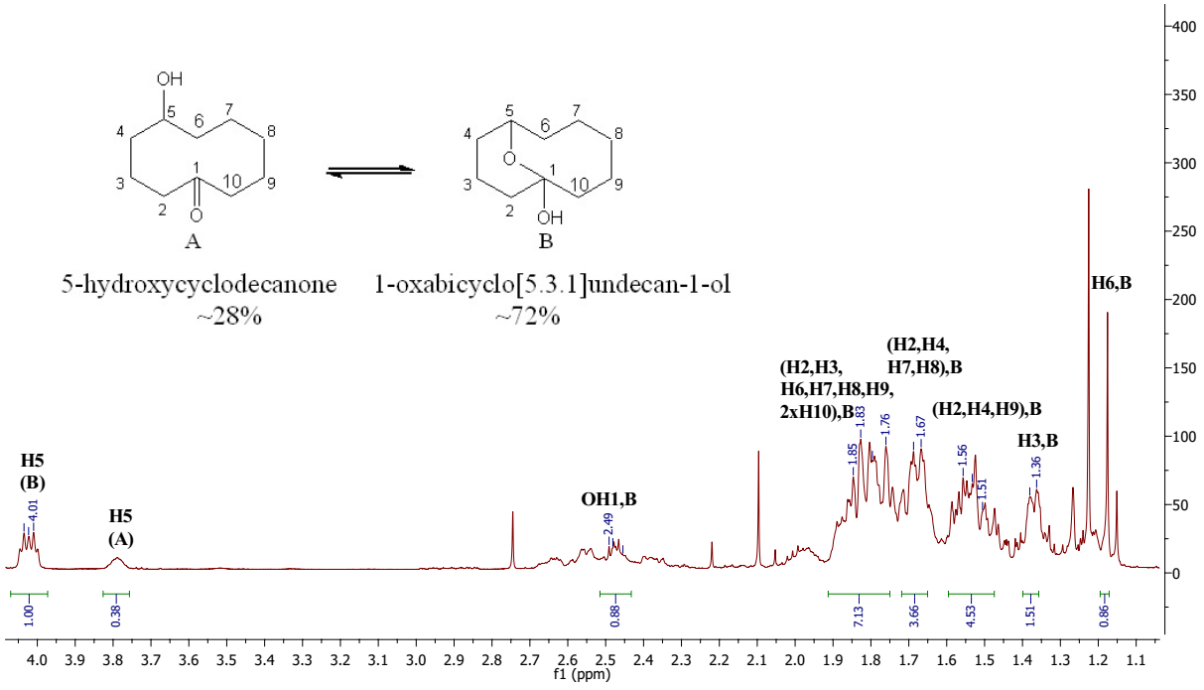


Figure S5 vi [1]H NMR of 1-oxabicyclo[5.3.1]undecan-1-ol (72%; B; CYP101B1). The other minor product 5-hydroxycyclodecanone (A) was also present (28%) in the NMR (not all the hydrogens of this minor product are labelled). The H5 (A) peak of 5-hydroxycyclodecanone at 3.79 ppm was highlighted in the figure above[7].

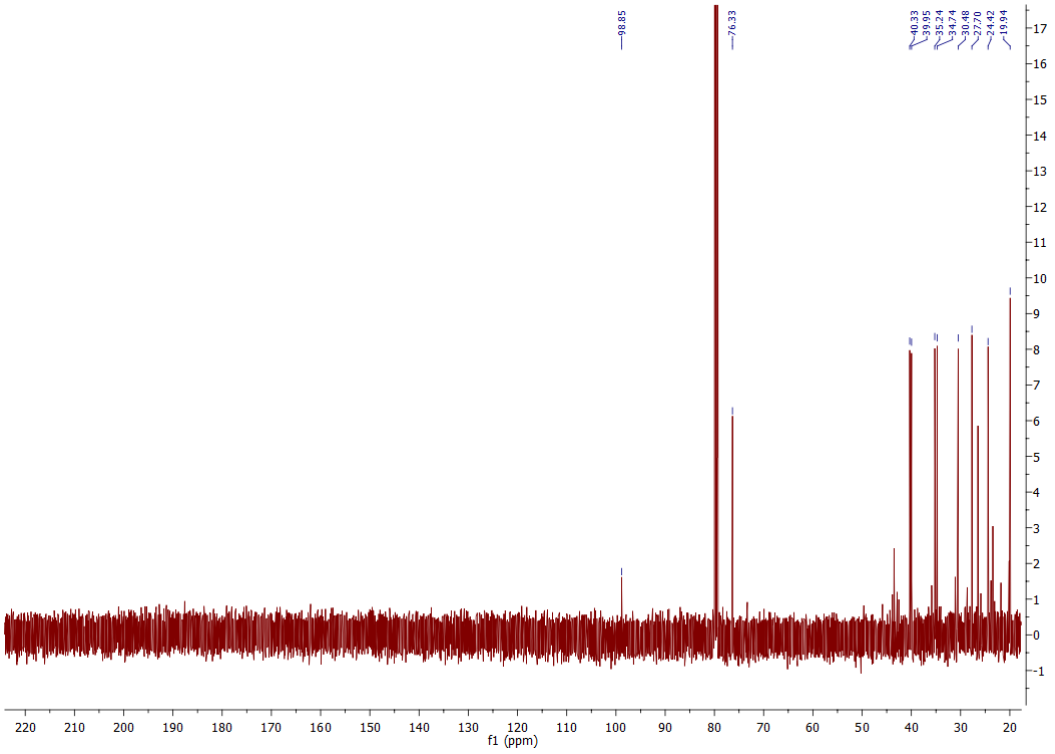


Figure S5 vii [13]C NMR of 1-oxabicyclo[5.3.1]undecan-1-ol [7].

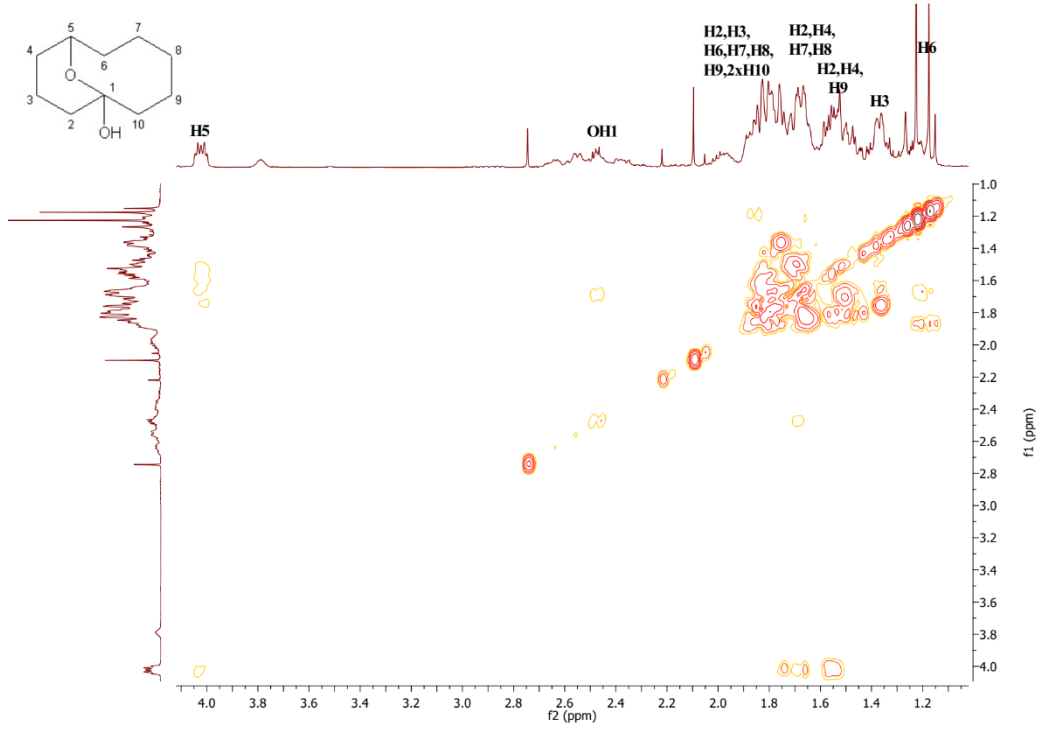


Figure S5 viii gCOSY NMR of 1-oxabicyclo[5.3.1]undecan-1-ol.

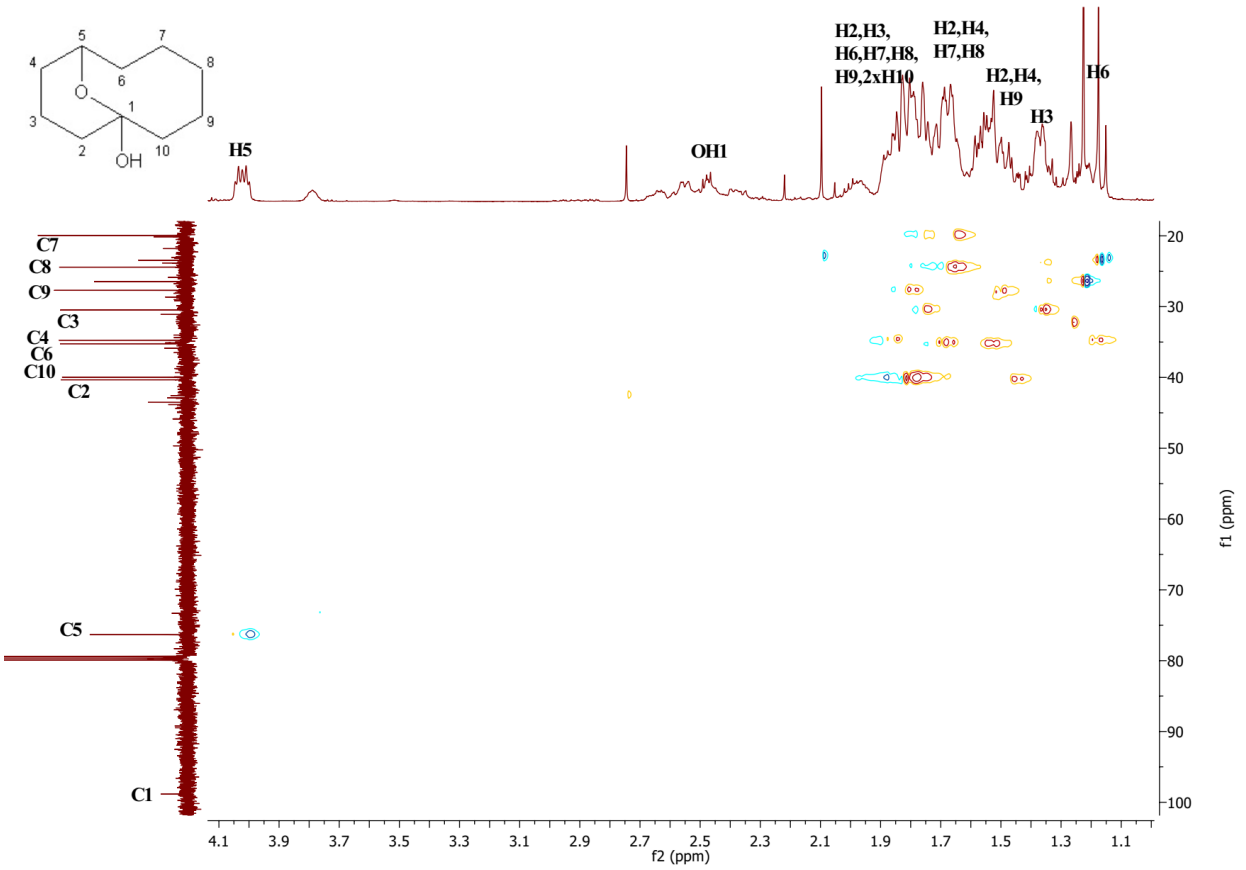


Figure S5 ix HSQC NMR of 1-oxabicyclo[5.3.1]undecan-1-ol.

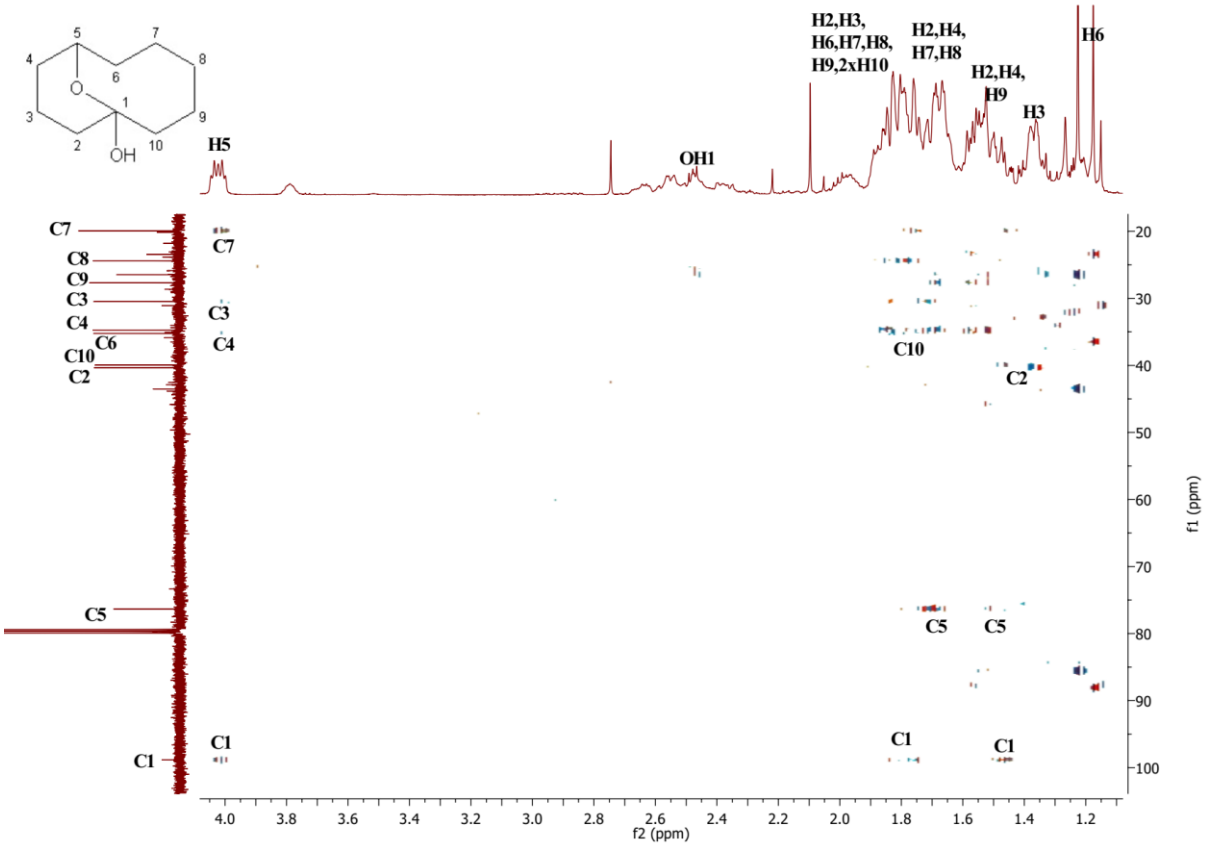


Figure S5 x Expansion (18 ppm to 104 ppm region) of the HMBC NMR of 1-oxabicyclo[5.3.1]undecan-1-ol. The product was confirmed as an oxabicycloalkanol as ^{13}C and HMBC NMR did not contains a carbonyl peak. Instead a characteristic signal of was observed at 98.85 ppm which displayed a correlation with H5 (3.97-4.07 ppm) in the HMBC NMR. The product was further confirmed by comparing the NMR with that reported NMR in the literature [7].

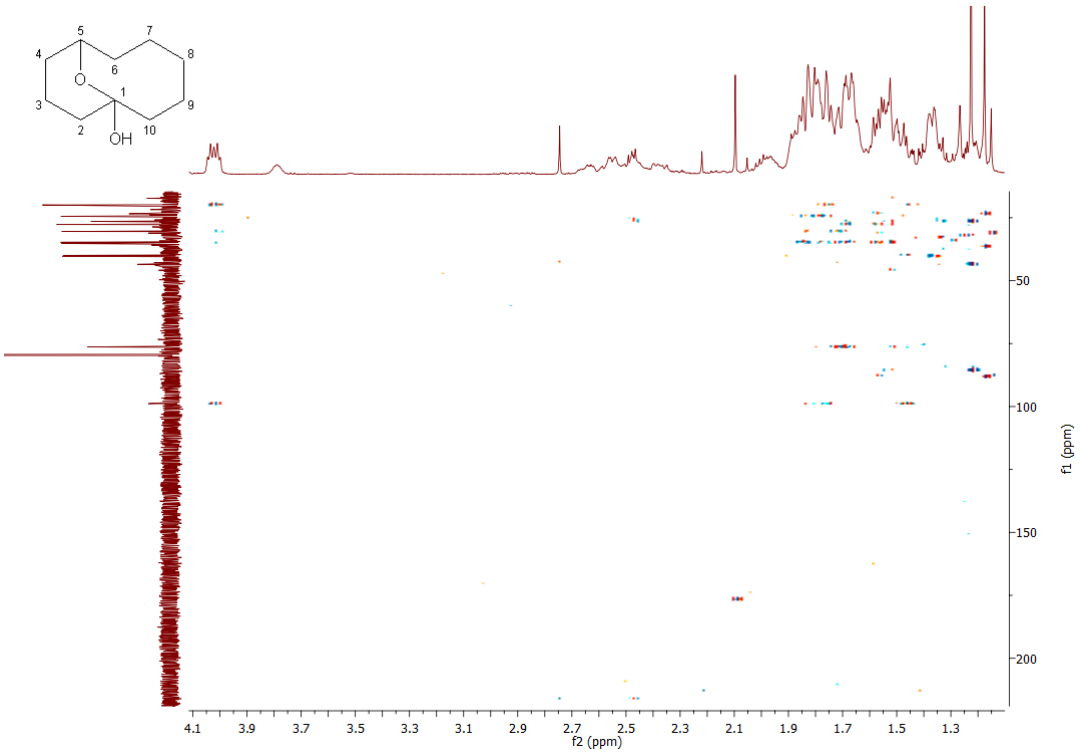


Figure S5 xi HMBC NMR of 1-oxabicyclo[5.3.1]undecan-1-ol.

1-oxabicyclo[6.3.1]undecan-1-ol

^{1}H NMR (600 MHz, $CDCl_3$) δ 4.13-4.01 (m, 1H, H6; B), 3.9-3.79 (m, 1H, H6; A), 2.70-1.59 (m, 2H, 2xH2; A), 2.41-2.33 (m, 2H, 2xH10; A), 2.01-1.87 (m, 4H, 2xH2; B & 2xH3; A), 1.80-1.72 (m, 8H, 2xH5; B, 2xH7; B, 2xH10; B & 2xH9; A), 1.7-1.61 (m, 4H, 2xH4; B & 2xH8; B), 1.63-1.46 (m, 12H, 2xH3; B, 2xH9; B, 2xH4; A, 2xH5; A, 2xH7; A & 2xH8; A)

^{13}C NMR (151 MHz, $CDCl_3$) δ 217.20 (C1; A), 105.08 (C1; B), 78.08 (C6; B), 72.20 (C6; A), 44.54 (C2 & C10; A), 43.18 (C2 & C10; B), 36.47 (C5 & C7; A&B), 27.57 (C4 & C8; B), 25.93 (C3 & C9; A), 25.36 (C4 & C8; A, C3 & C9; B).

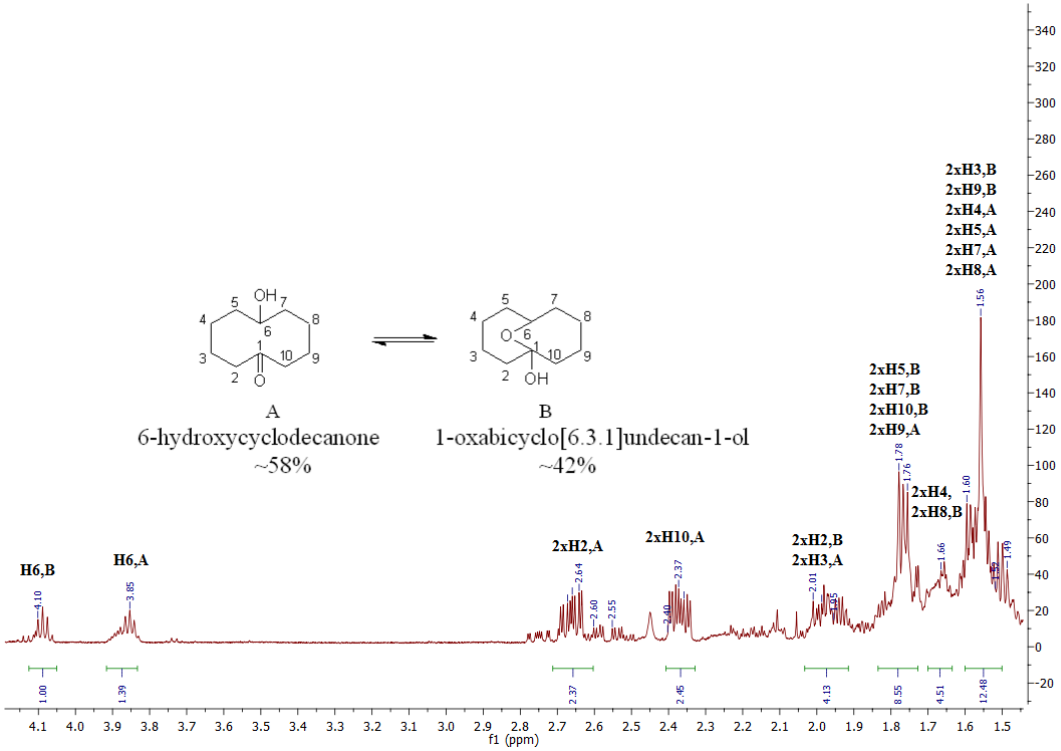
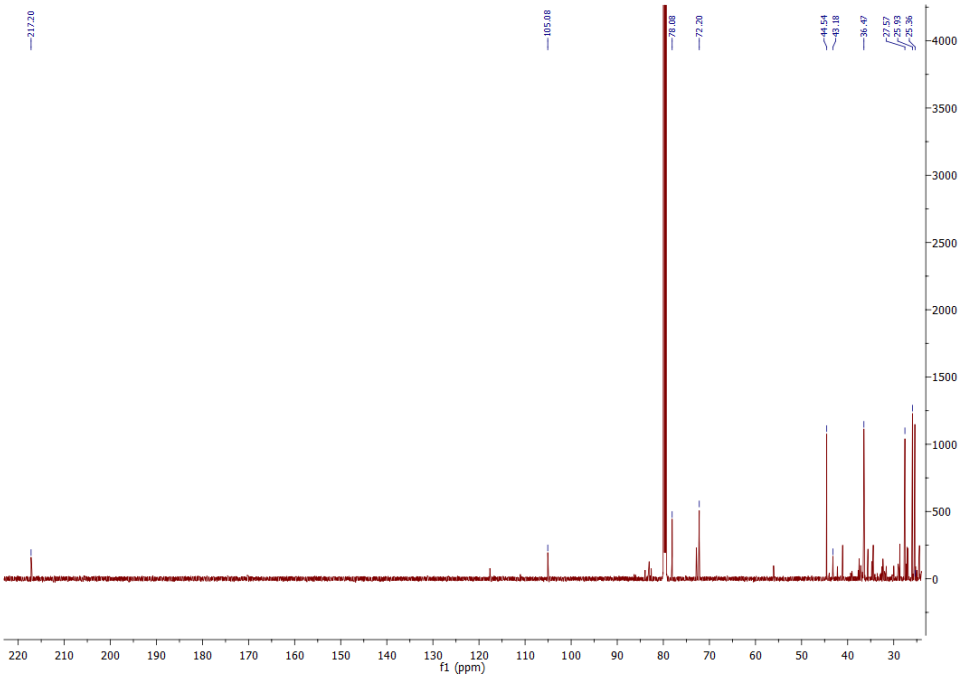
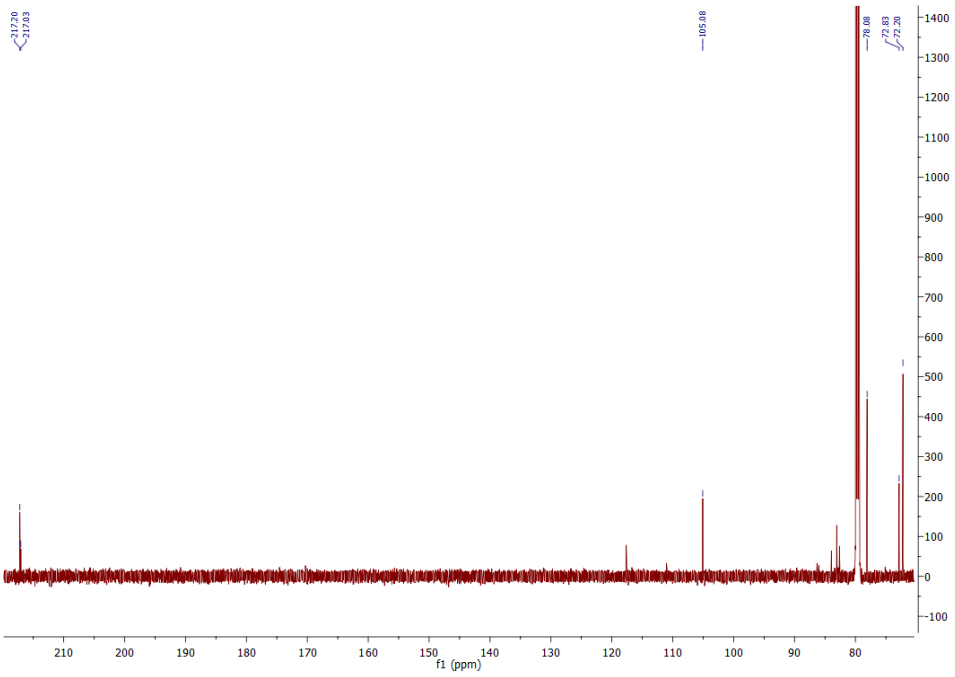


Figure S5 xii ^{1}H NMR of the 1-oxabicyclo[6.3.1]undecan-1-ol and 6-hydroxycyclodecanone mixture. The H6; B and H6; A signals were confirmed by comparing the reported ^{1}H NMR in literature (Table S2 below) [7].



(a)



(b)

Figure S5 xiii (a) ^{13}C NMR of 1-oxabicyclo[6.3.1]undecan-1-ol and 6-hydroxycyclodecanone mixture [7]. **(b)** Expansion of the ^{13}C NMR (70 ppm to 220 ppm region) to highlight the minor metabolite signals that was assigned as 4-hydroxy by using HMBC NMR below.

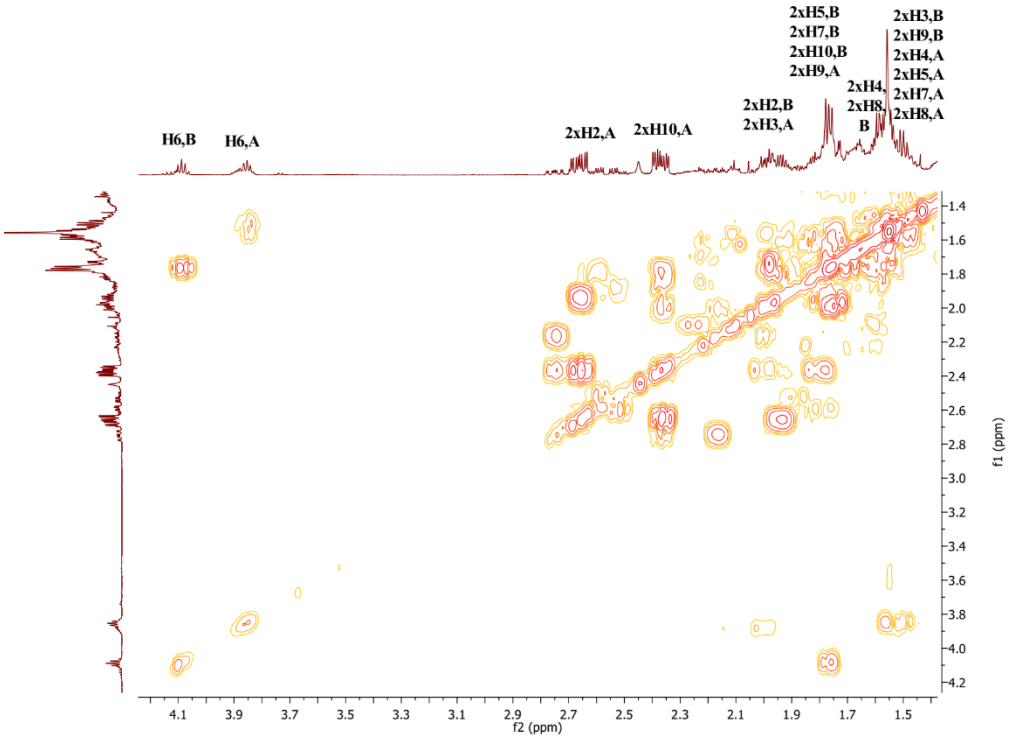


Figure S5 xiv gCOSY NMR of 1-oxabicyclo[6.3.1]undecan-1-ol and 6-hydroxycyclodecanone mixture.

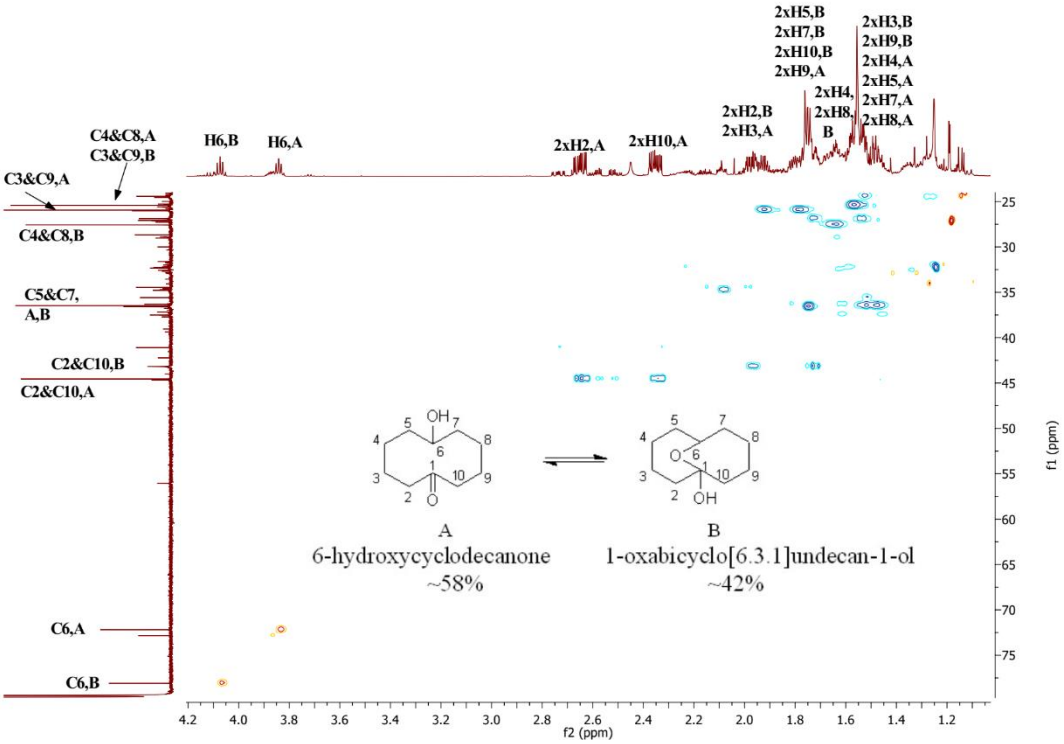
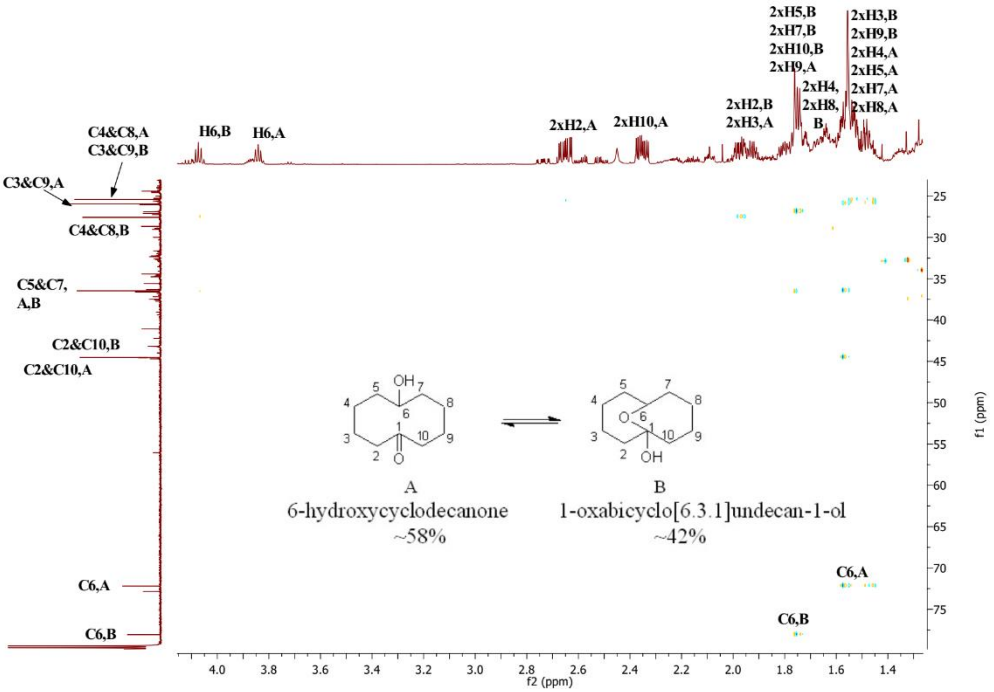
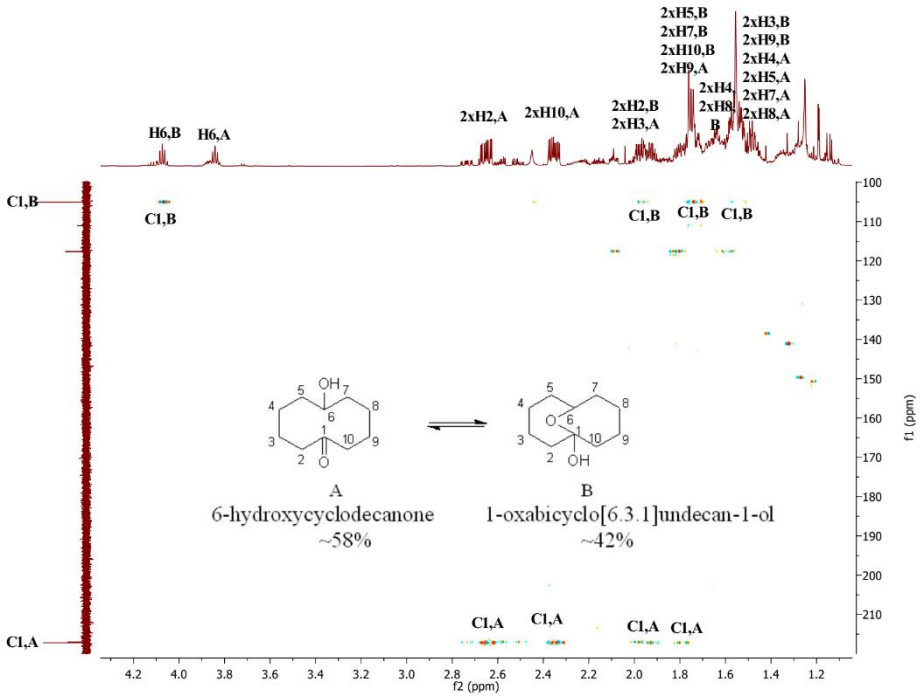


Figure S5 xv HSQC NMR of 1-oxabicyclo[6.3.1]undecan-1-ol and 6-hydroxycyclodecanone mixture.

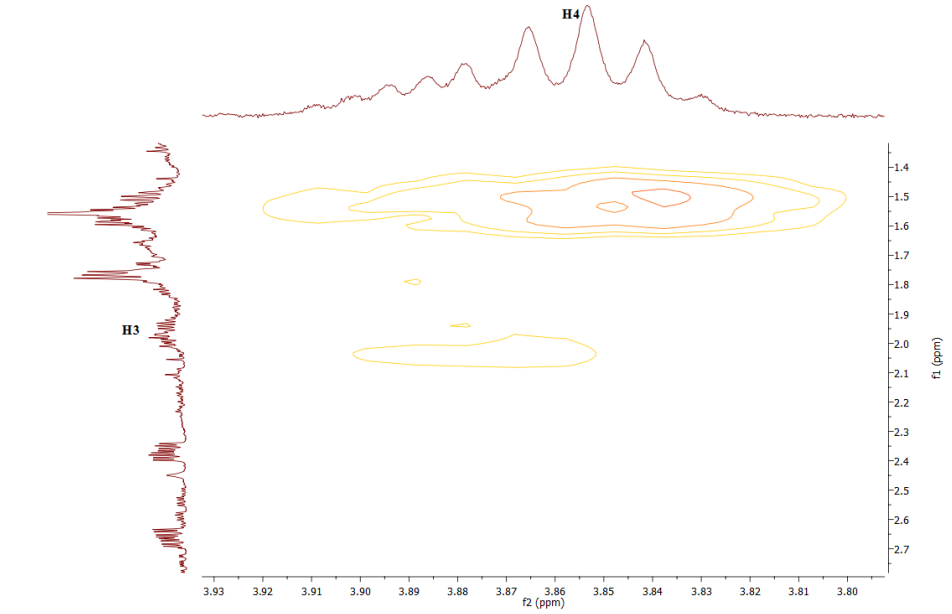


(a)

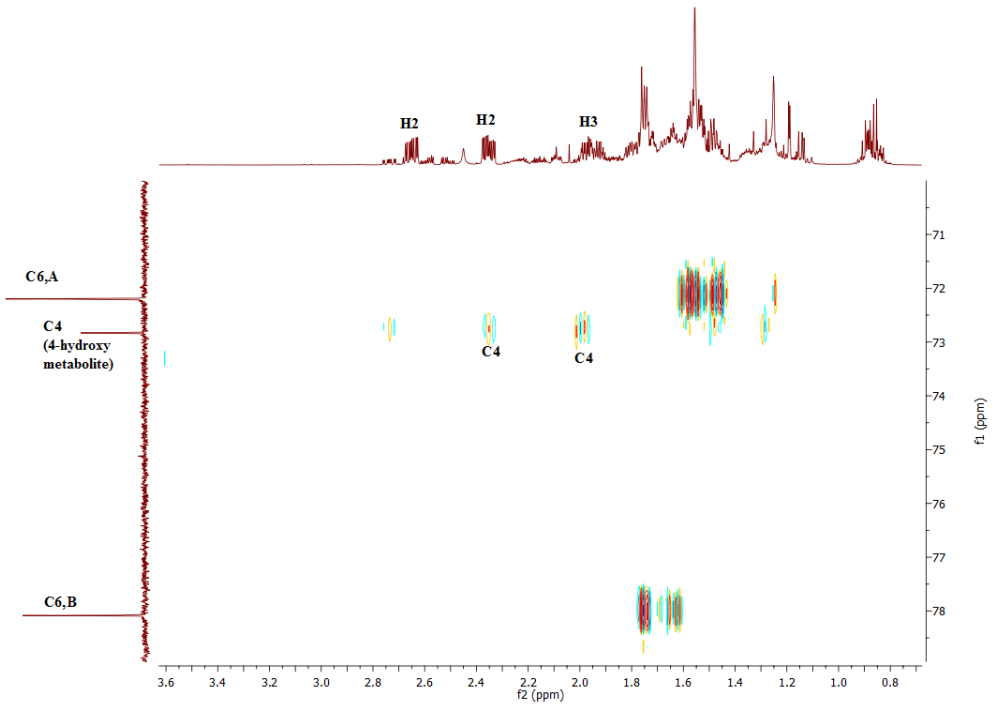


(b)

Figure S5 xvi (a) Expansion (20 ppm to 80 ppm region) of the HMBC NMR of 1-oxabicyclo[6.3.1]undecan-1-ol and 6-hydroxycyclodecanone mixture. **(b)** Expansion (100 ppm to 220 ppm region) of the HMBC NMR of 1-oxabicyclo[6.3.1]undecan-1-ol and 6-hydroxycyclodecanone which highlighted the correlations of C1,A and C1,B with the protons.



(a)



(b)

Figure S5 xvii (a) Expansion of the gCOSY NMR to highlight the interaction of H4 with H3. **(b)** Expansion of the HMBC NMR to highlight the peak of a possible 4-hydroxy metabolite which was assigned by the correlations of C4 (72.83) with the protons of 2.41-2.33 ppm and 2.01-1.87 ppm. The product was not fully characterised (only ~2% of total product).

Table S2 ^{1}H NMR signals of the CH-OH and HC-O groups in $CDCl_3$ [7].

Hydroxy ketone	**CH-OH (C) (δ)**	**Hemiacetal**	**HC-O (C) (δ)**
5-hydroxycyclodecanone	3.75 (69.9)	1-oxabicyclo[5.3.1] undecan-1-ol	4.0 (73.6)
6-hydroxycyclodecanone	3.83 (69.9)	1-oxabicyclo[6.3.1] undecan-1-ol	4.07 (76.6)

7-hydroxycyclododecanone

^{1}H NMR of 7-hydroxycyclododecanone [8] δ 3.75-3.65 (m,1H, H7), 2.81-2.68 (m, 2H, H2 & H12), 2.25-2.11 (m, 2H, H2 & H12), 1.92-1.8 (m, 2H, H3 & H11), 1.68-1.51 (m, 4H, H3, H6, H8 & H11), 1.49-1.39 (m, 6H, H4, H5, H6, H8, H9 & H10), 1.37-1.29 (m, 2H, H5 & H9), 1.27-1.17 (m, 2H, H4 & H10).

^{13}C NMR (126 MHz, $CDCl_3$) δ 215.27 (C1), 71.43 (C7), 42.96 (C2 & C12), 36.62 (C6 & C8), 27.58 (C4 & C10), 25.37 (C5 & C9), 25.0 (C3 & C11).

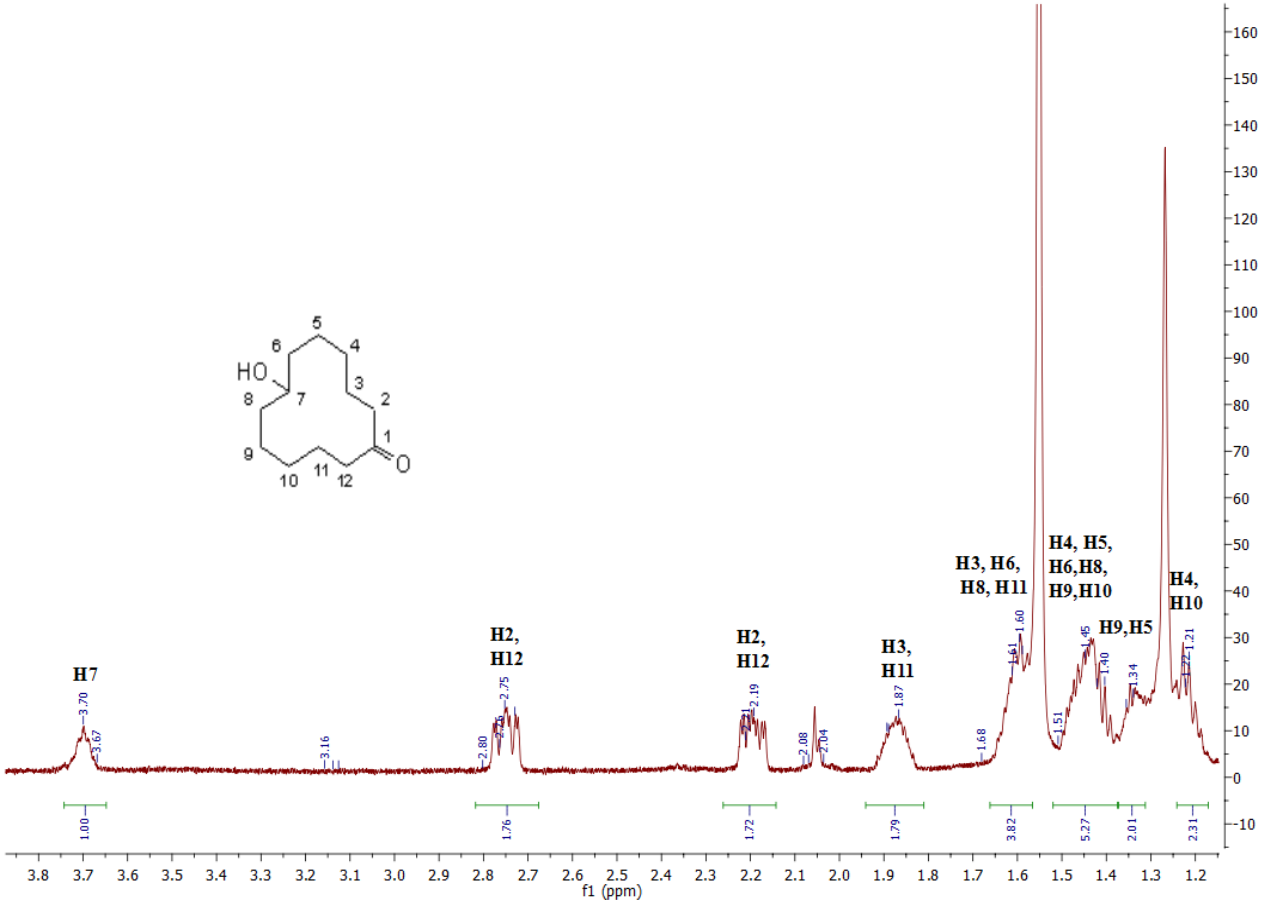


Figure S5 xviii ^{1}H NMR of 7-hydroxycyclododecanone.

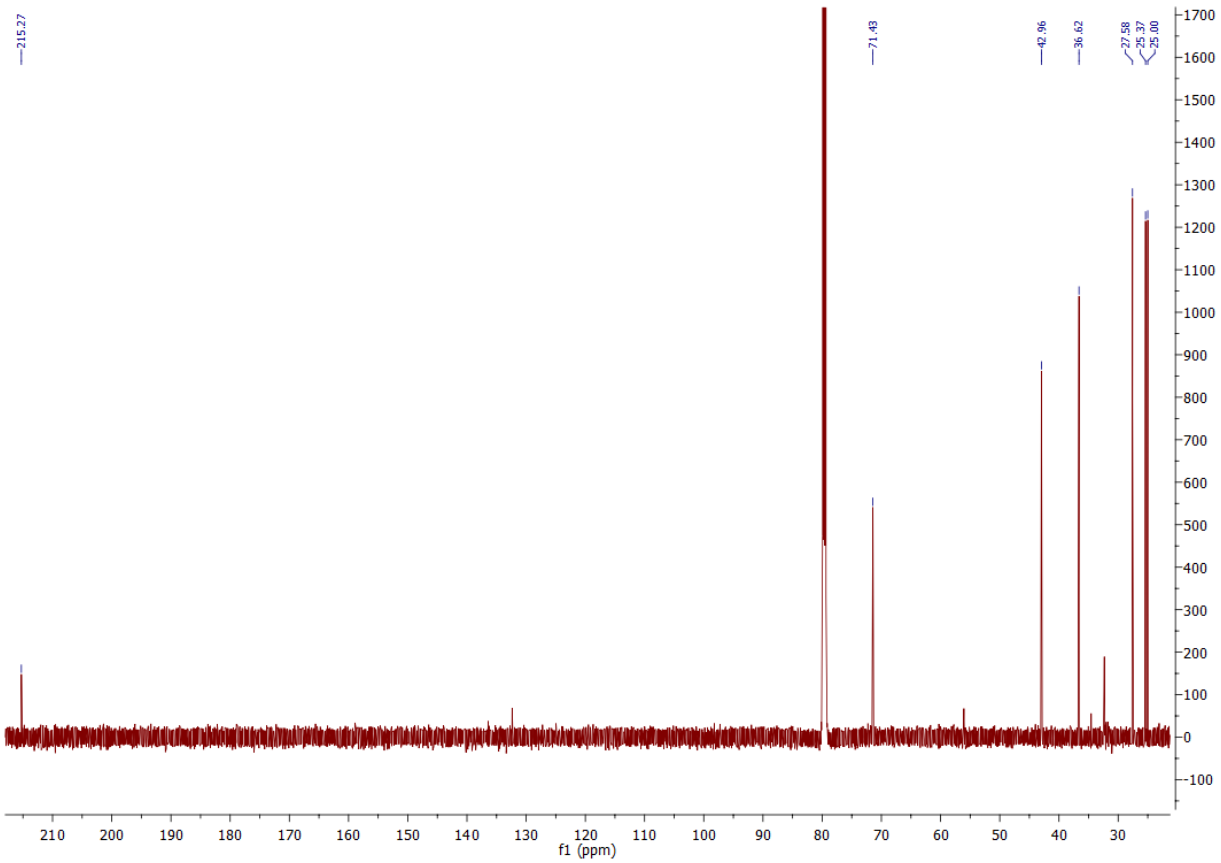


Figure S5 xix ^{13}C NMR of 7-hydroxycyclododecanone.

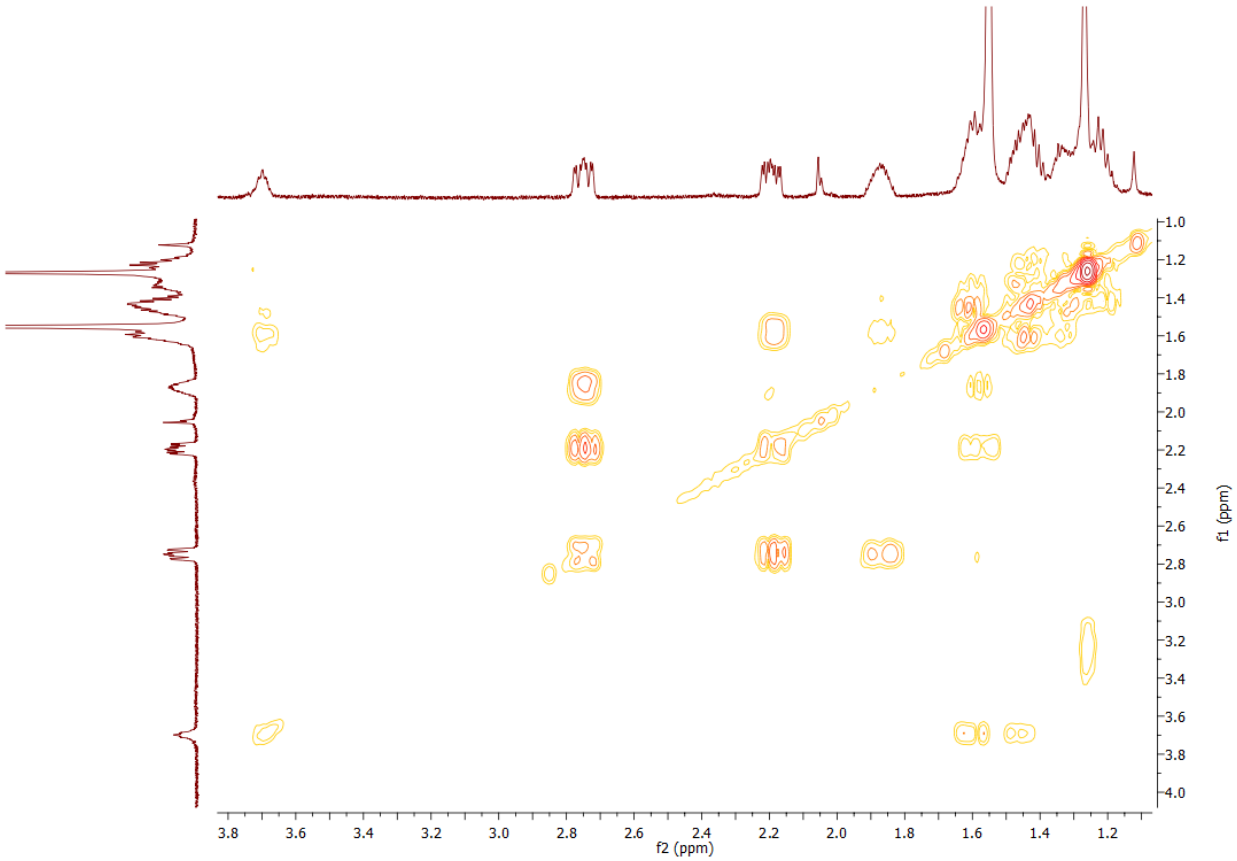


Figure S5 xx gCOSY NMR of 7-hydroxycyclododecanone.

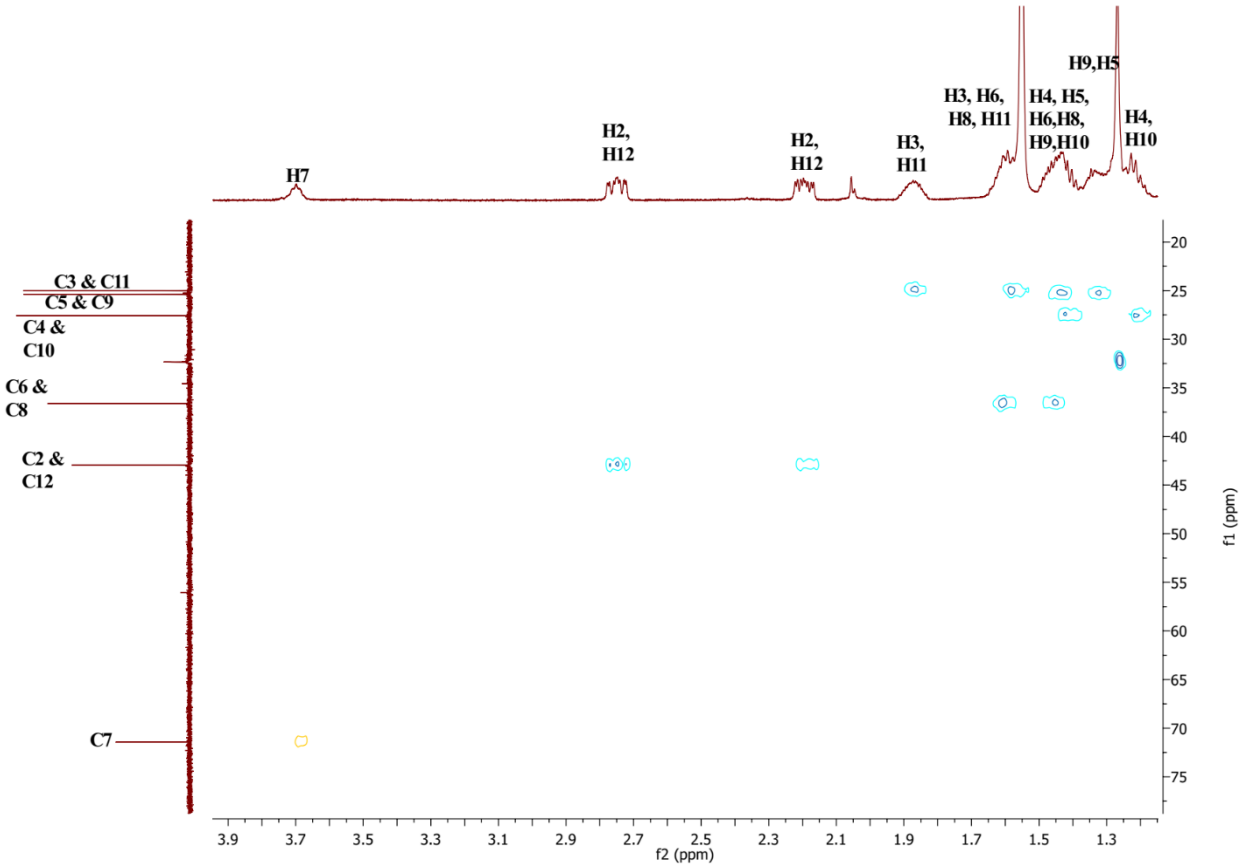


Figure S5 xxi HSQC NMR of 7-hydroxycyclododecanone.

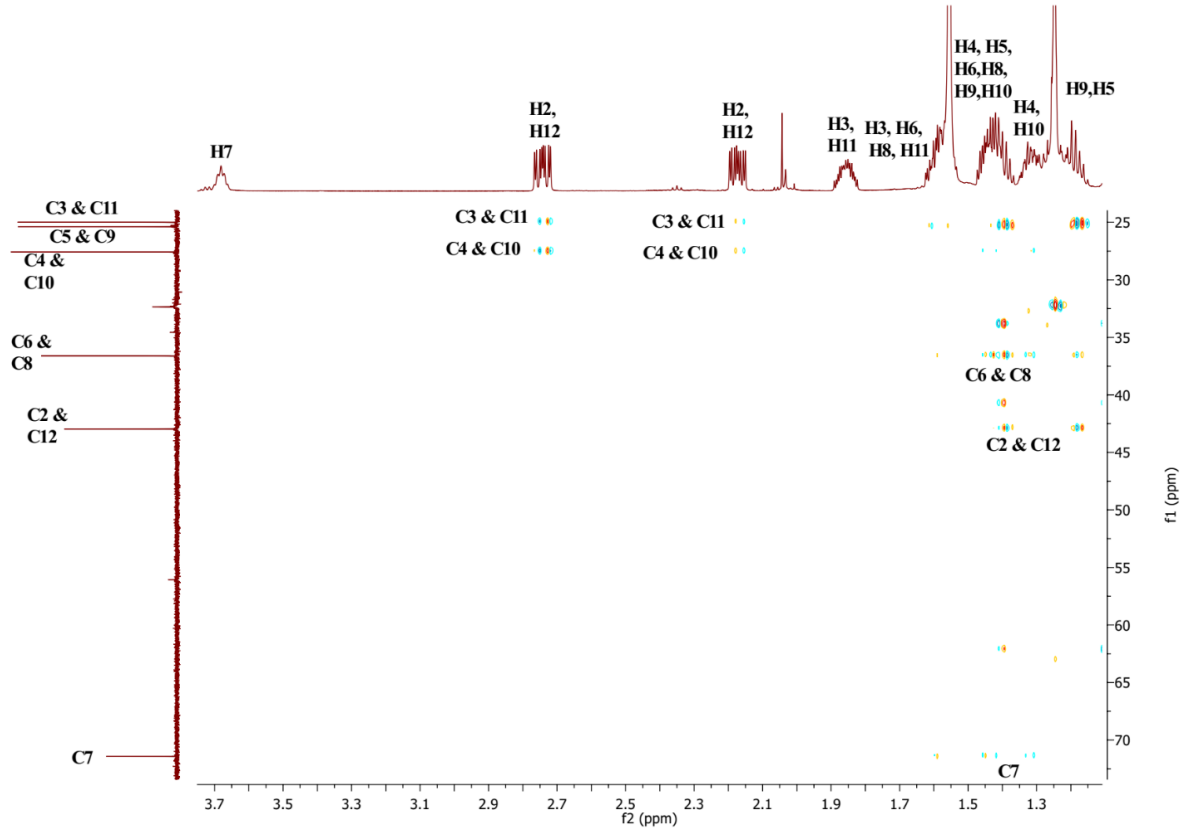


Figure S5 xxii HMBC NMR of 7-hydroxycyclododecanone zoomed in on the 20 to 76 ppm region.

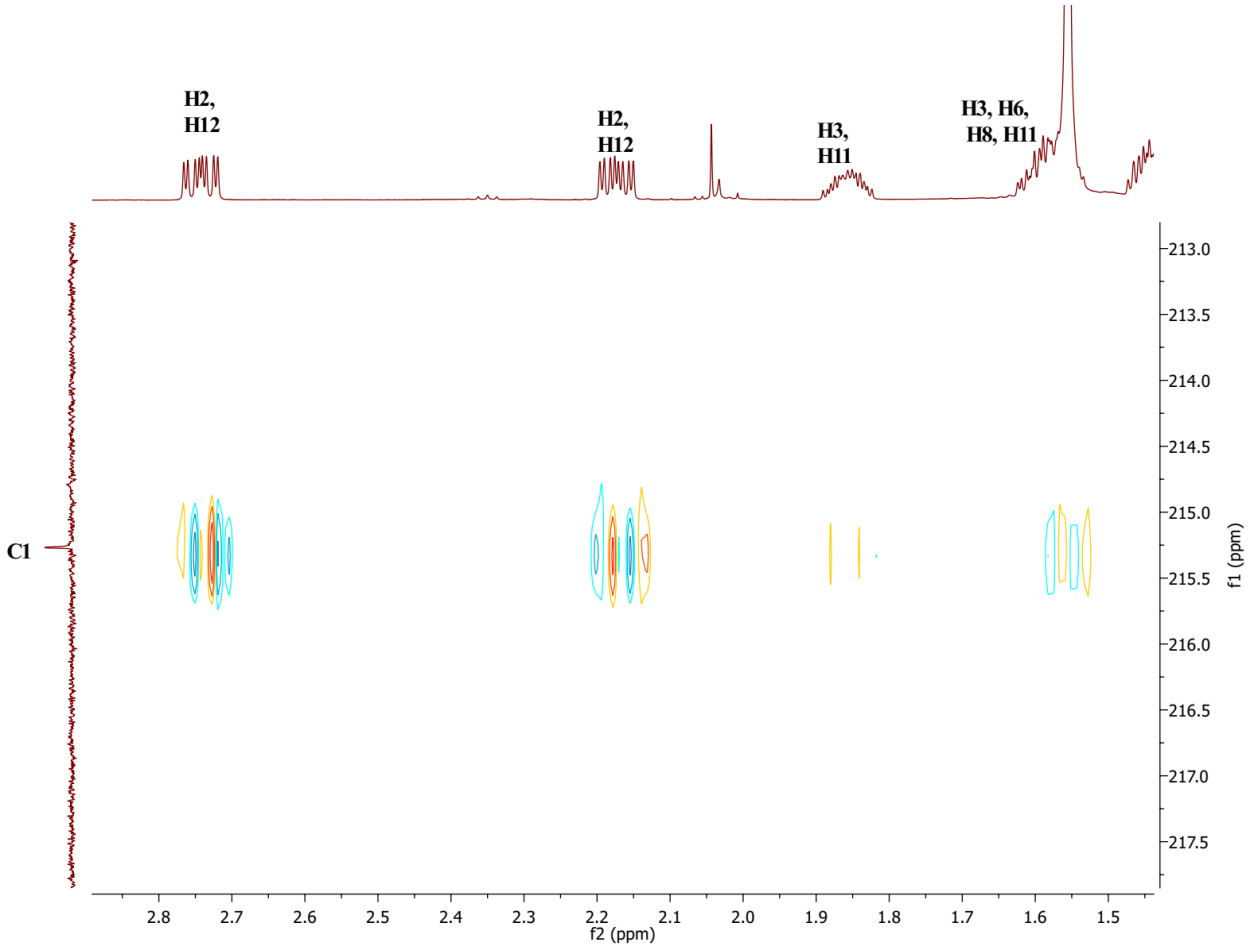


Figure S5 xxiii HMBC NMR of 7-hydroxycyclododecanone zoomed in on the C=O region, which highlights the interaction of C1 (215.27 ppm) with H2 and H12 (2.81-2.68 ppm & 2.25-2.11 ppm) and with H3 and H11 (1.92-1.8 ppm & 1.68-1.51 ppm).

NMR data for *trans*-4-hydroxycyclohexyl acetate:

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.77-4.70 (m, 1H, H1), 3.76-3.68 (m, 1H, H4), 2.04 (s, 3H, 3xH8), 2.02-1.93 (m, 4H, $H2_{eq}$, $H3_{eq}$, $H5_{eq}$ & $H6_{eq}$), 1.54-1.36 (m, 4H, $H2_{ax}$, $H3_{ax}$, $H5_{ax}$ & $H6_{ax}$).

^{13}C NMR (126 MHz, $CDCl_3$) δ 173.27 (C7), 74.38 (C1), 71.66 (C4), 34.94 (C3 & C5), 31.27 (C2 & C6), 24.01 (C8).

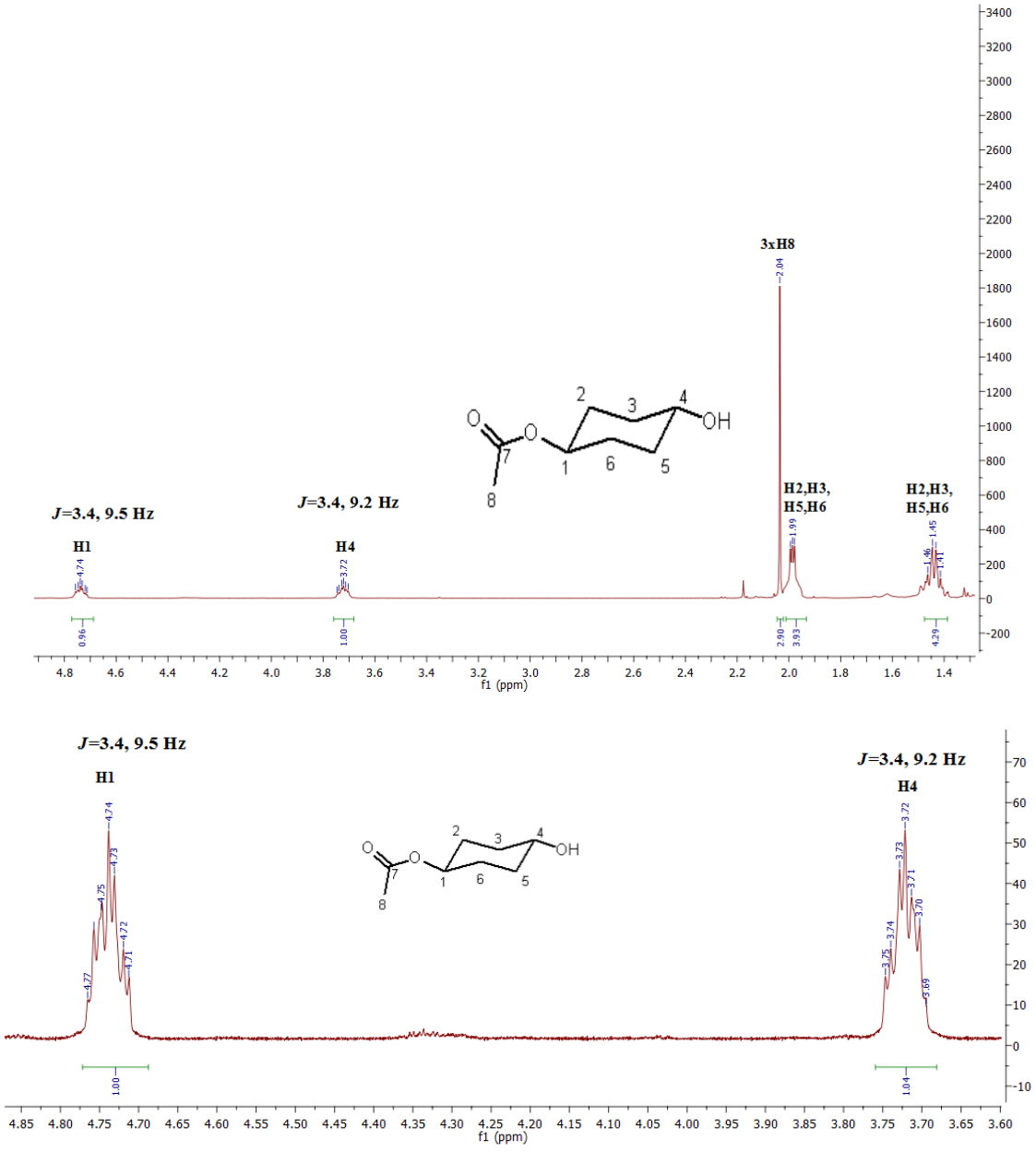


Figure S5 xxiv ^{1}H NMR of *trans*-4-hydroxycyclohexyl acetate.

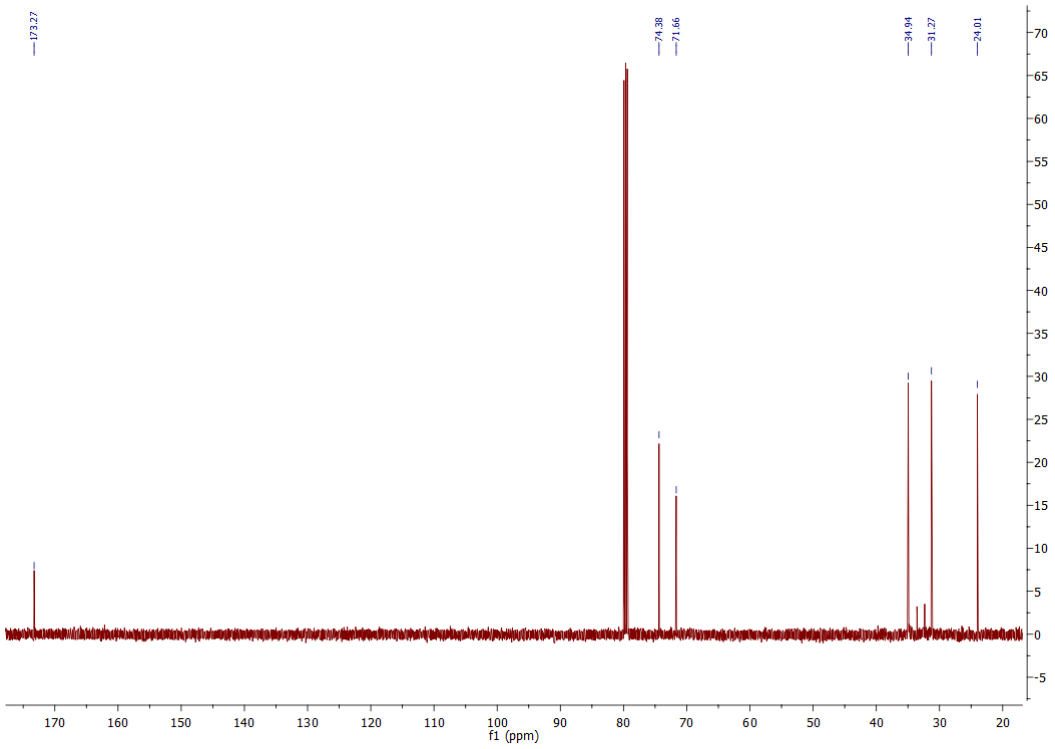


Figure S5 xxv ^{13}C NMR of *trans*-4-hydroxycyclohexyl acetate.

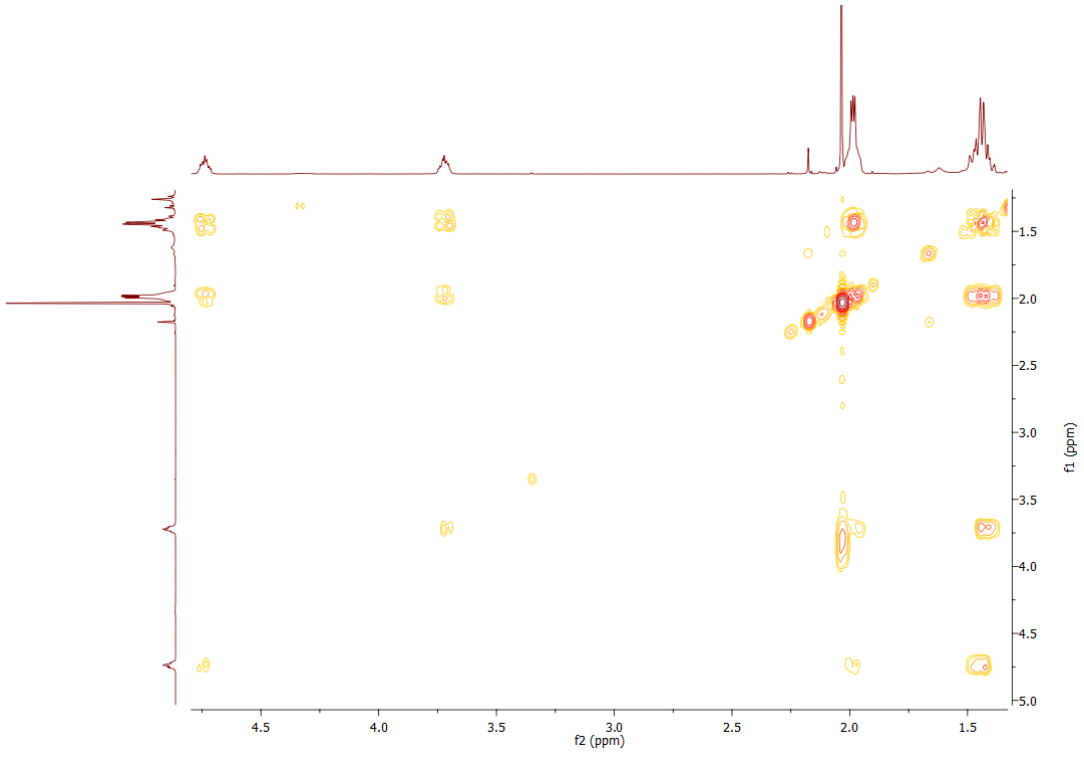


Figure S5 xxvi gCOSY NMR of *trans*-4-hydroxycyclohexyl acetate.

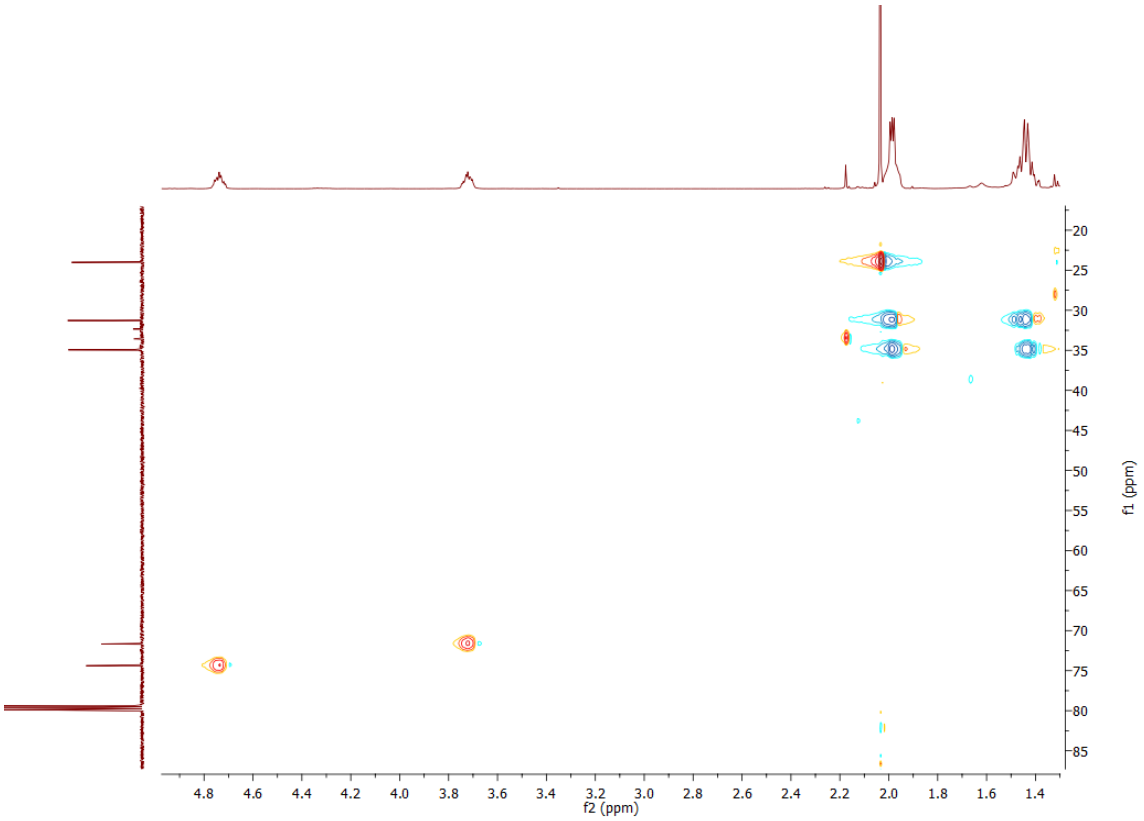


Figure S5 xxvii HSQC NMR of *trans*-4-hydroxycyclohexyl acetate.

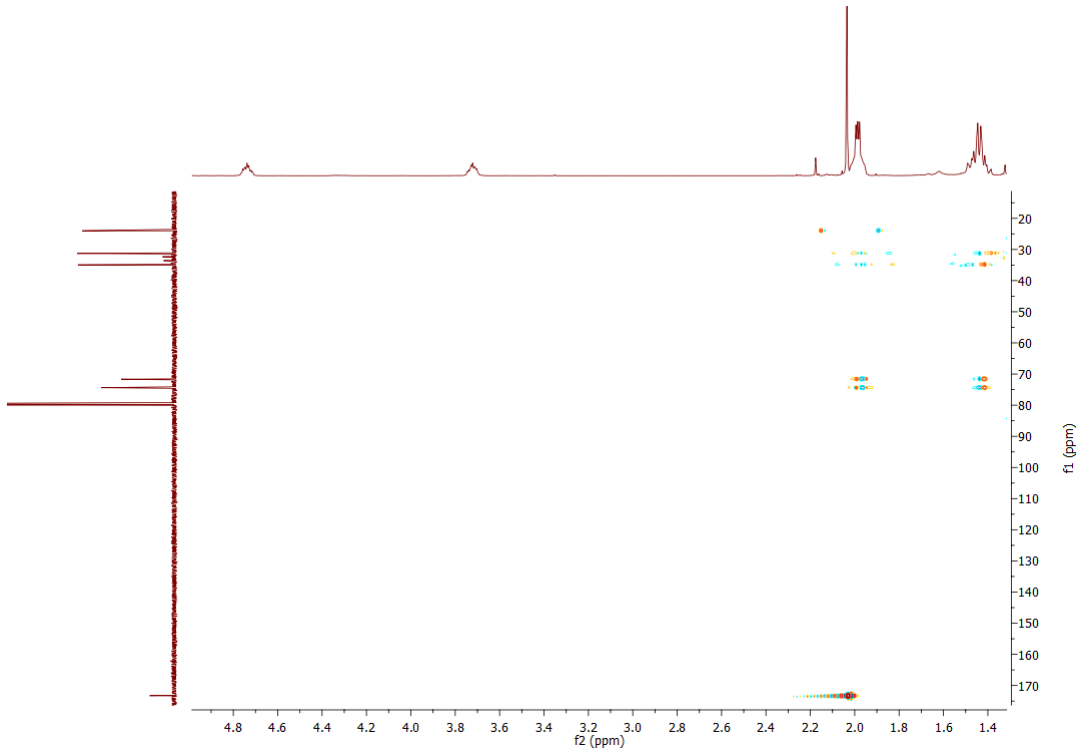
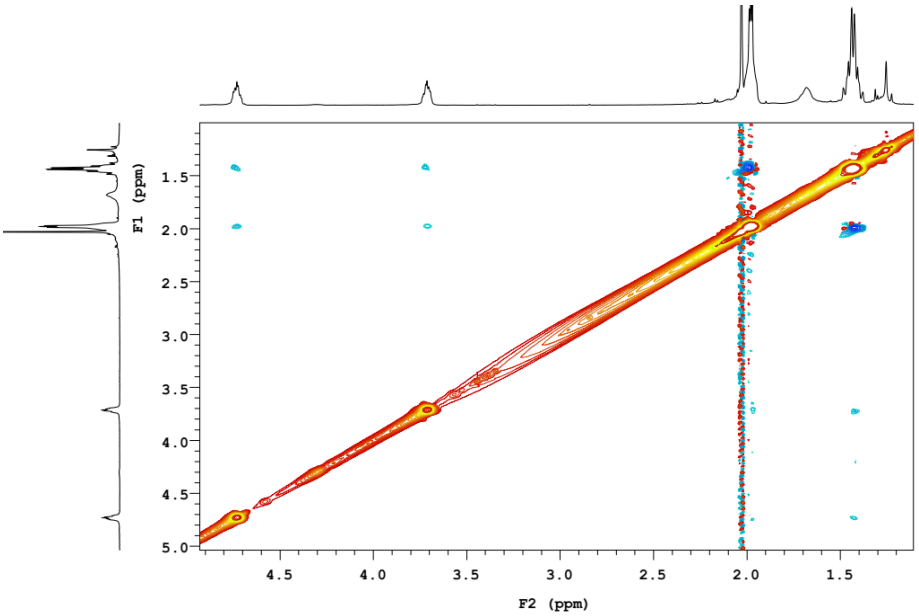
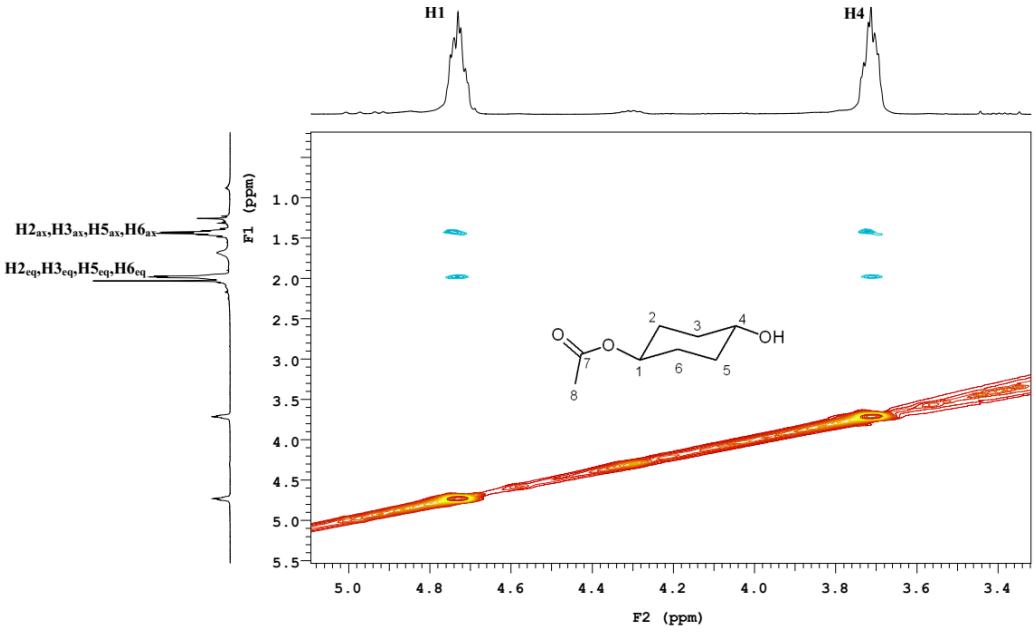


Figure S5 xxviii HMBC NMR of *trans*-4-hydroxycyclohexyl acetate.



(a)



(b)

Figure S5 xxix (a) ROESY NMR of *trans*-4-hydroxycyclohexyl acetate. **(b)** Expansion of the ROESY NMR highlighting the interactions of H1 (4.77-4.70 ppm) and H4 (3.76-3.72 ppm) with the protons of (2.02-1.93 ppm) and (1.54-1.36 ppm) region.

Data for *trans*-4-hydroxycyclohexyl butyrate:

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.78-4.66 (m, 1H, H1), 3.71-3.60 (m, 1H, H4), 2.22 (*t*, *J* = 7.4 Hz, 2H, 2xH8), 1.99-1.88 (m, 4H, $H2_{eq}$, $H3_{eq}$, $H5_{eq}$ & $H6_{eq}$), 1.61 (*sextet*, *J* = 7.4 Hz, 2H, 2xH9), 1.47-1.33 (4H, $H2_{ax}$, $H3_{ax}$, $H5_{ax}$ & $H6_{ax}$), 0.91 (*t*, *J* = 7.4 Hz, 3H, 3xH10)

^{13}C NMR (126 MHz, $CDCl_3$) δ 175.93 (C7), 74.11(C1), 71.47 (C4), 39.17 (C8), 34.80 (C3 & C5), 31.25 (C2 & C6), 21.16 (C9), 16.22 (C10).

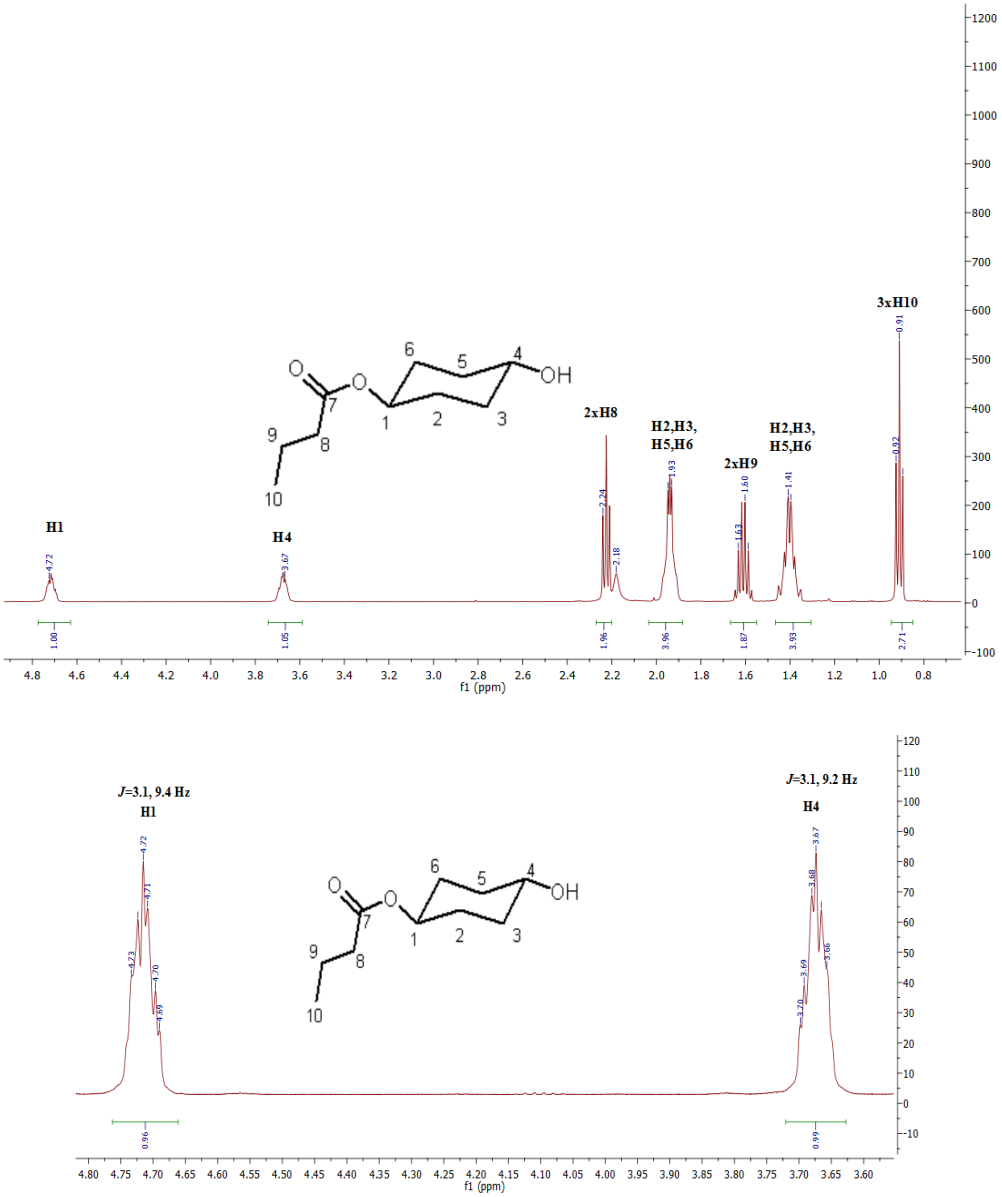


Figure S5 xxx ^{1}H NMR of *trans*-4-hydroxycyclohexyl butyrate.

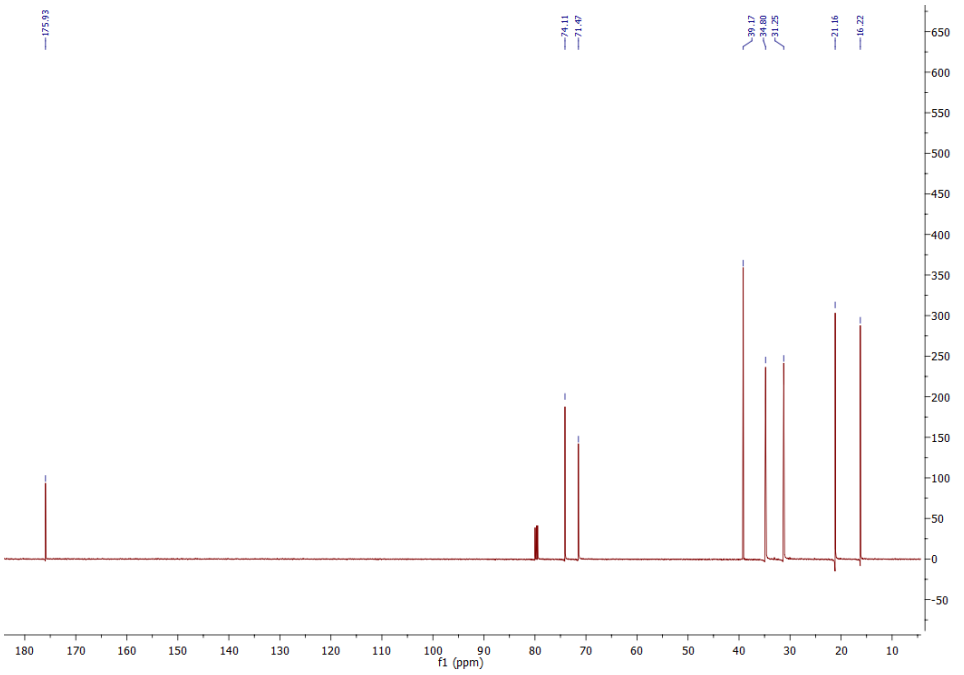


Figure S5 xxxi ^{13}C NMR of *trans*-4-hydroxycyclohexyl butyrate.

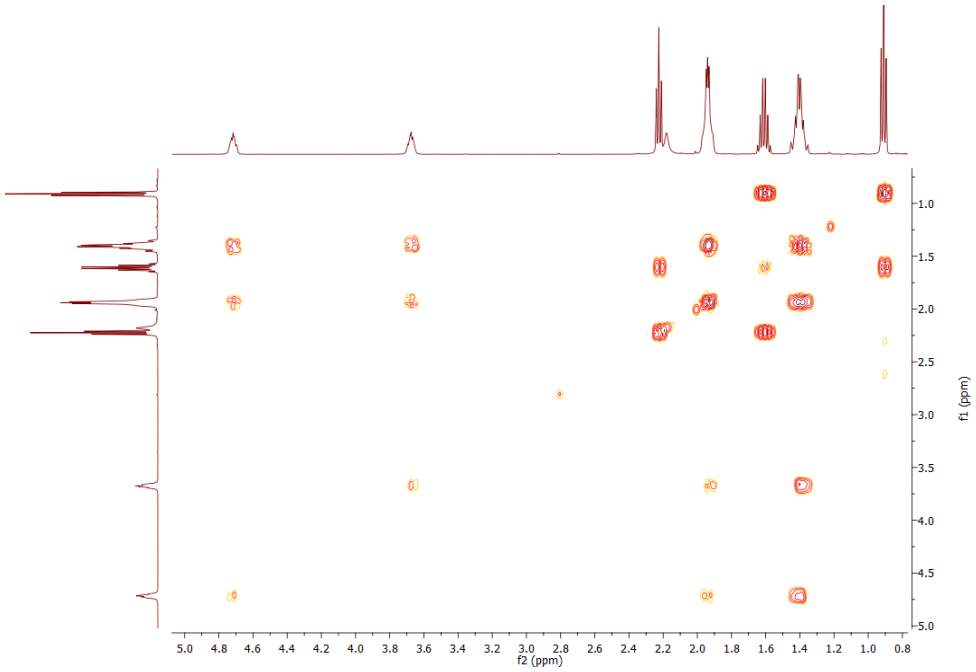


Figure S5 xxxii gCOSY NMR of *trans*-4-hydroxycyclohexyl butyrate.

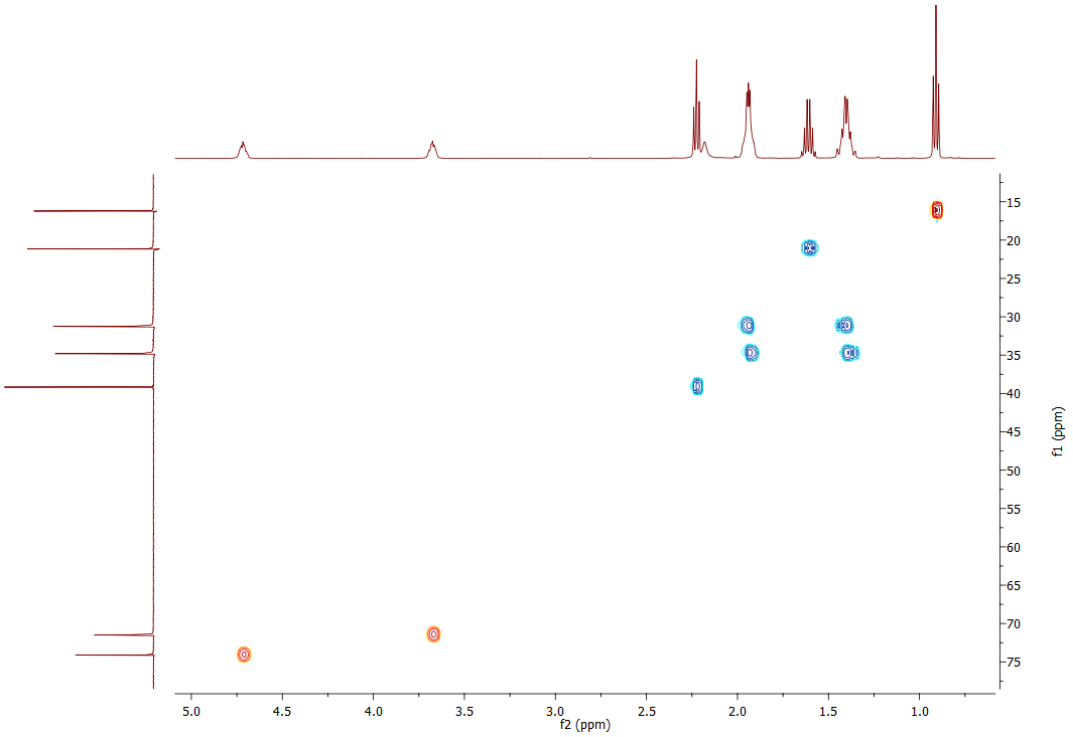
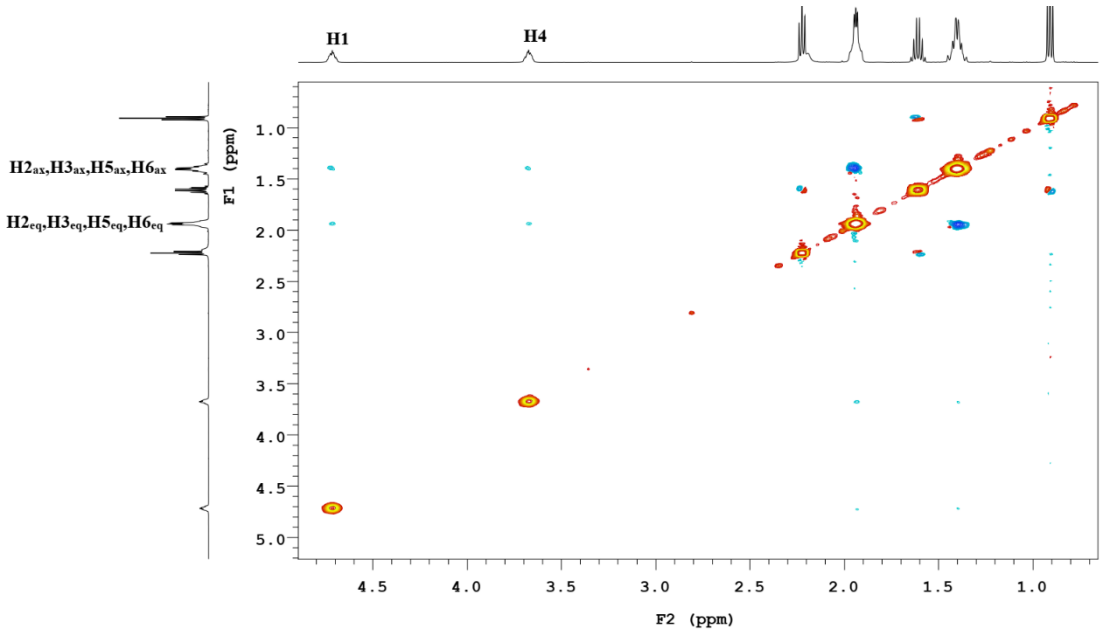
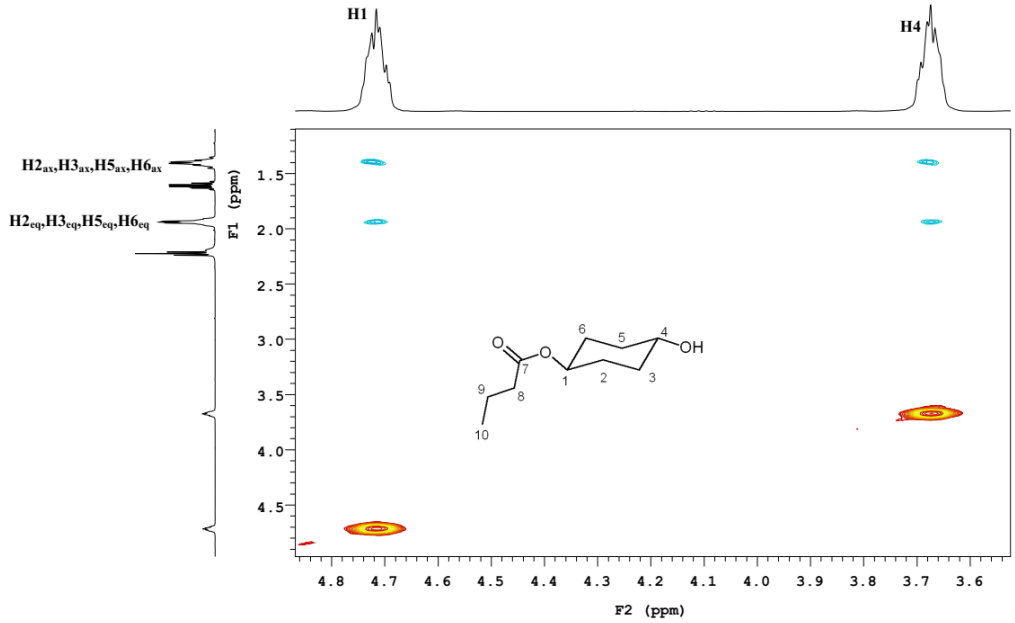


Figure S5 xxxiii HSQC NMR of *trans*-4-hydroxycyclohexyl butyrate.



(a)



(b)

Figure S5 xxxiv (a) ROESY NMR of *trans*-4-hydroxycyclohexyl butyrate. **(b)** Expansion of the ROESY NMR which highlighted the interactions of H1 (4.78-4.66 ppm) and H4 (3.71-3.60 ppm) with the protons of (1.99-1.88 ppm) and (1.47-1.33 ppm) region.

NMR for *trans*-4-hydroxycyclohexyl isobutyrate:

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.78-4.69 (m, 1H, H1), 3.78-3.7 (m, 1H, H3), 2.51 (*septet*, J = 7 Hz, 1H, H8), 2.04-1.89 (m, 4H, $H2_{eq}$, $H4_{eq}$, $H5_{eq}$ & $H6_{eq}$), 1.51-1.43 (m, 4H, $H2_{ax}$, $H4_{ax}$, $H5_{ax}$ & $H6_{ax}$), 1.16 (d, J = 7.0 Hz, 6H, 3xH9 & 3xH10)

^{13}C NMR (126 MHz, $CDCl_3$) δ 179.33 (C7), 73.82 (C1),71.60 (C3), 36.83 (C8), 34.75 (C3 & C5), 31.04 (C2 & C6), 21.64 (C9 & C10).

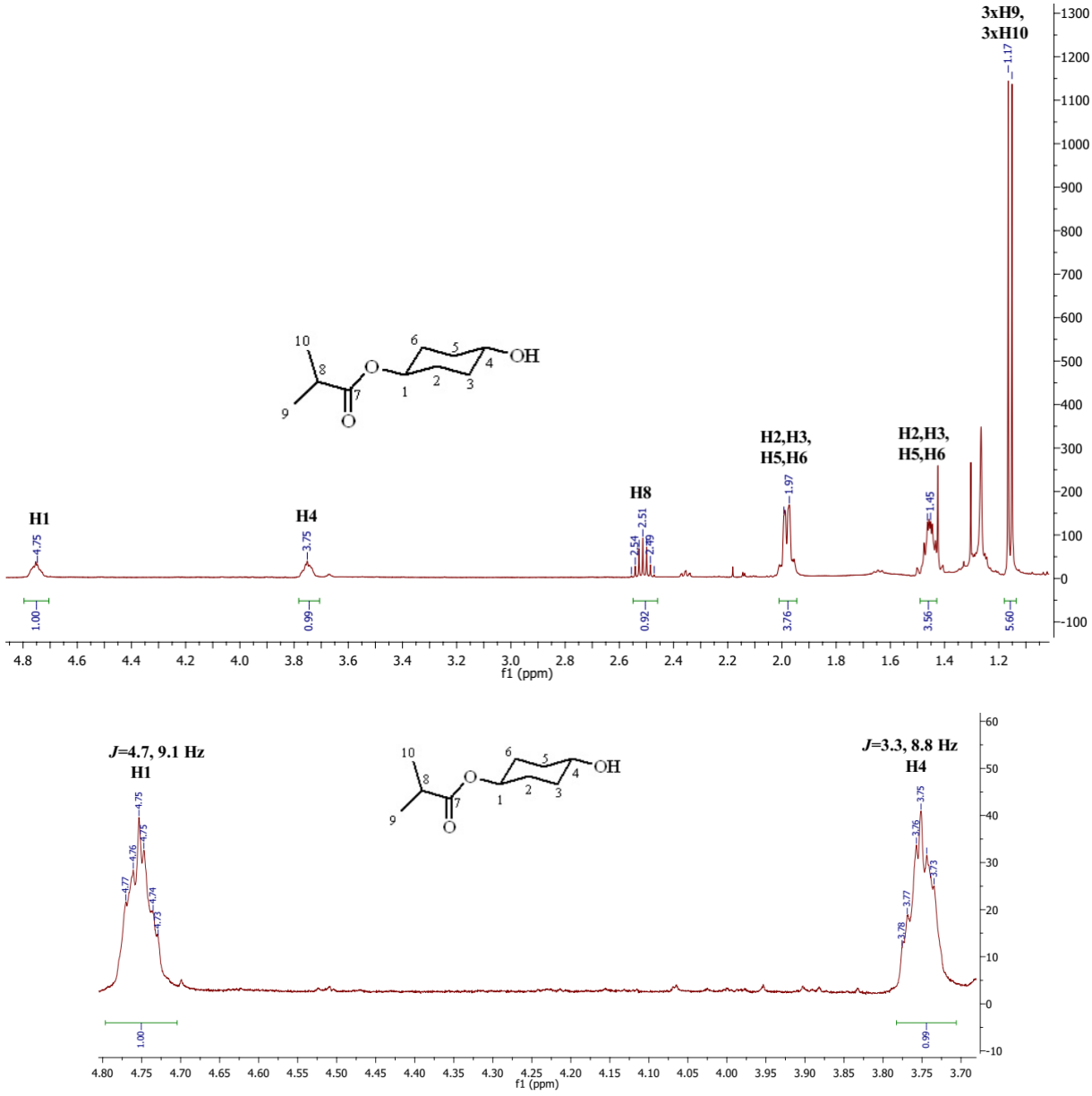


Figure S5 xxxv ^{1}H NMR of *trans*-4-hydroxycyclohexyl isobutyrate.

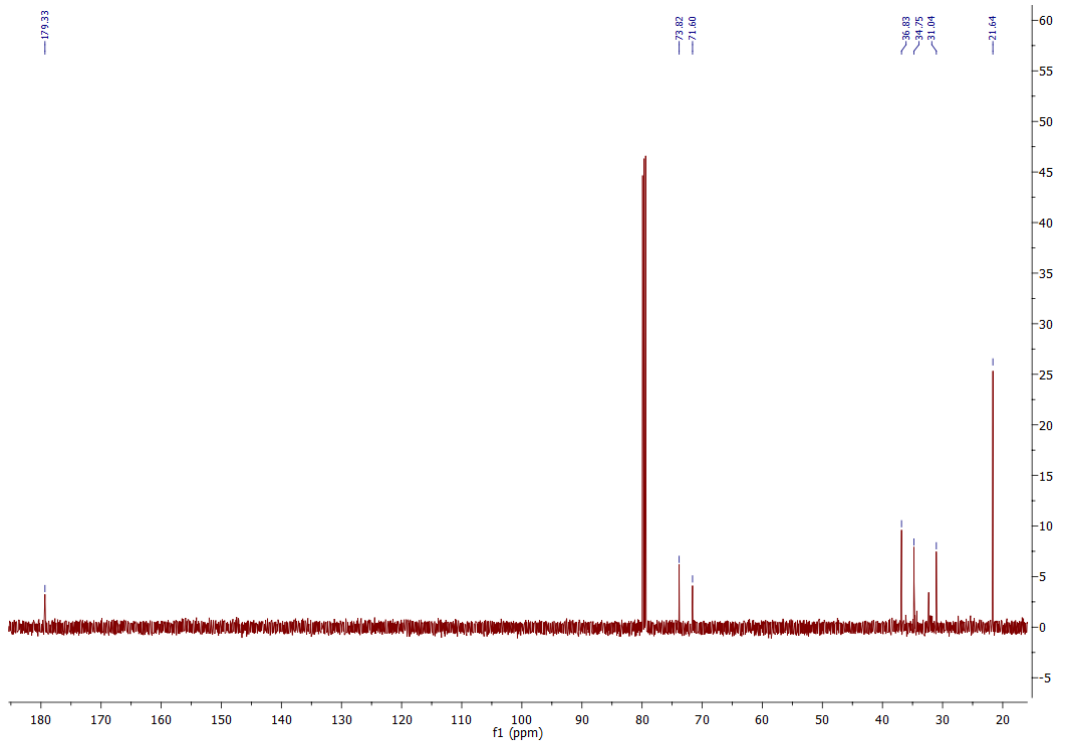


Figure S5 xxxvi ^{13}C NMR of *trans*-4-hydroxycyclohexyl isobutyrate.

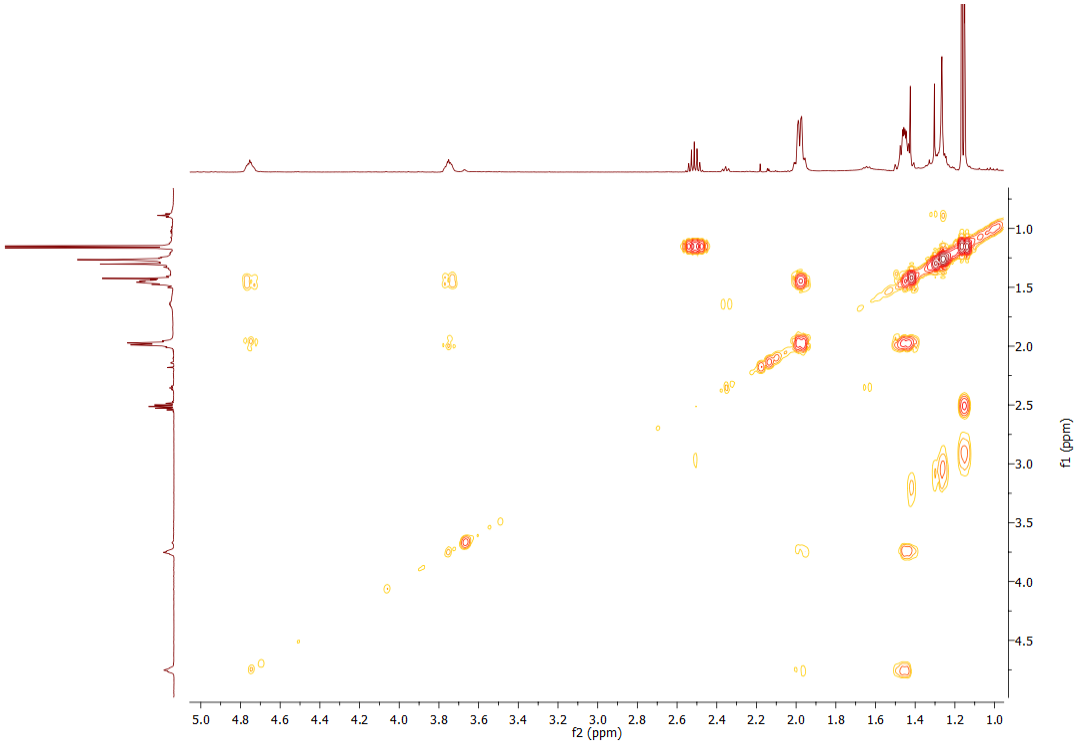


Figure S5 xxxvii gCOSY NMR of *trans*-4-hydroxycyclohexyl isobutyrate.

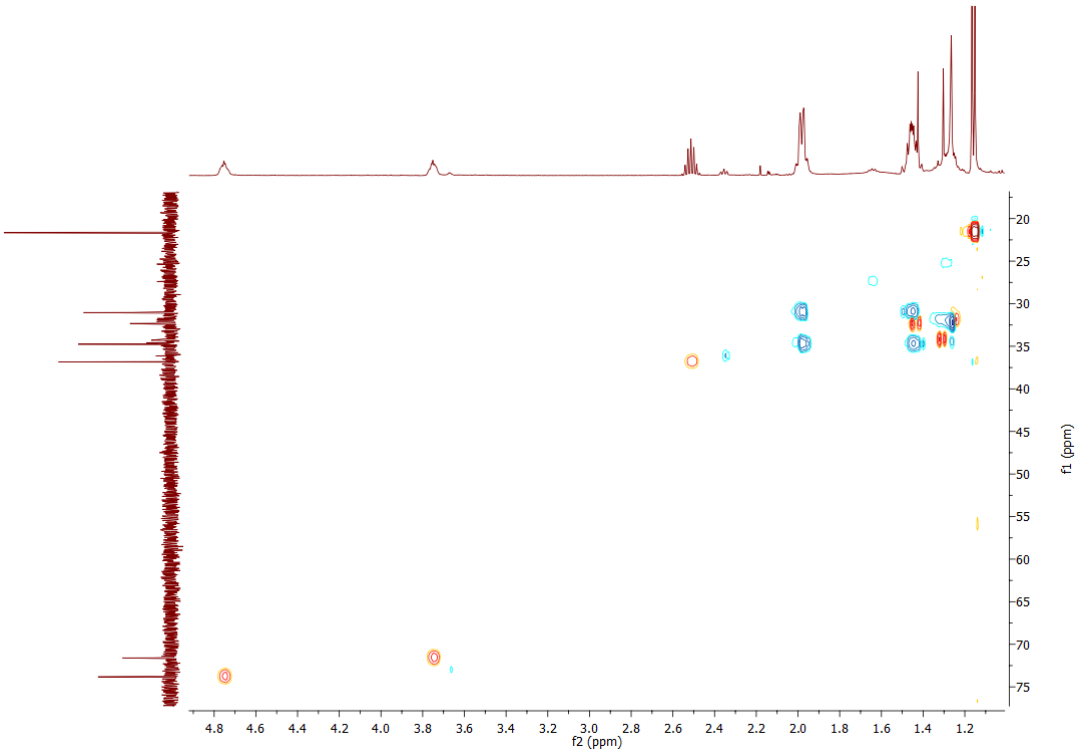


Figure S5 xxxviii HSQC NMR of *trans*-4-hydroxycyclohexyl isobutyrate.

NMR data for methyl (*trans*-4-hydroxycyclohexyl)acetate:

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.68 (s, 3H, 3xH9), 3.59-3.52 (m, 1H, H4), 2.21 (d, J = 6.8 Hz, 2H, 2xH7), 2.02-1.94 (m, 2H, $H3_{eq}$ & $H5_{eq}$), 1.84-1.74 (m, 3H, H1, $H2_{eq}$ & $H6_{eq}$), 1.36-1.28 (m, 2H, $H3_{ax}$ & $H5_{ax}$), 1.12-1.00 (m, 2H, $H2_{ax}$ & $H6_{ax}$).

^{13}C NMR (126 MHz, $CDCl_3$) δ 175.94 (C8), 73.25 (C4), 54.10 (C9), 43.71 (C7), 37.86 (C3 & C5), 36.51 (C1), 33.61 (C2 & C6).

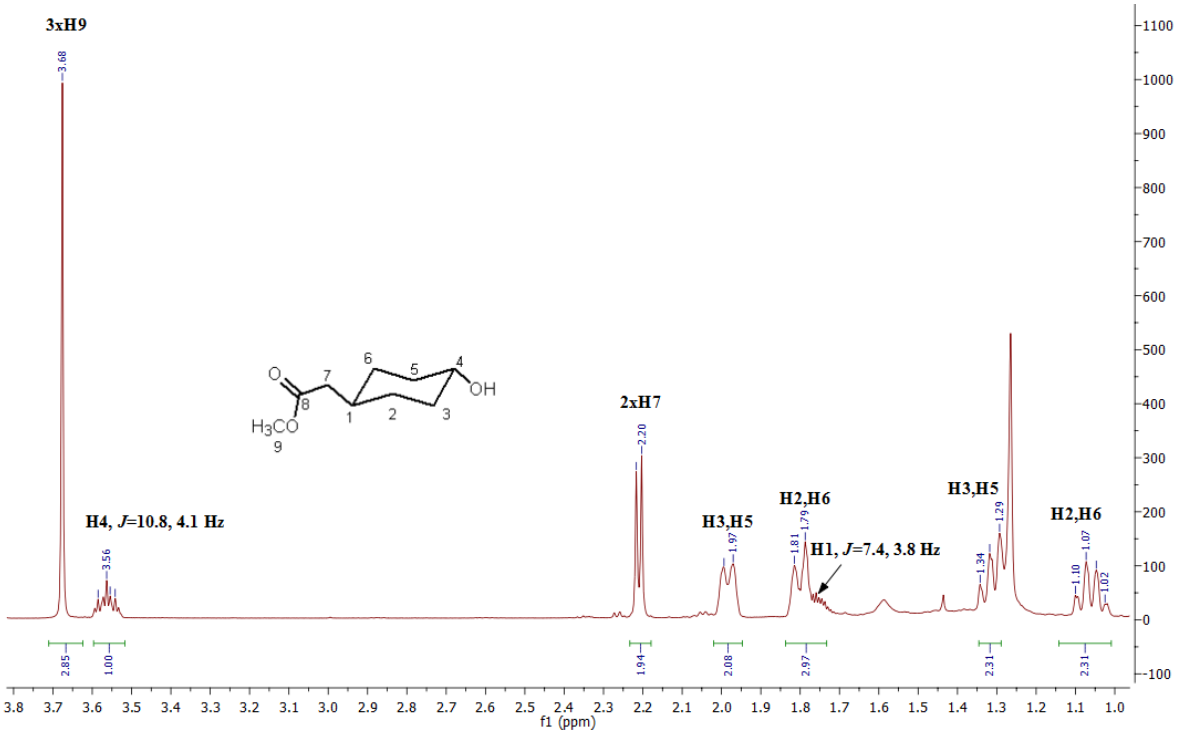


Figure S5 xxxix ^{1}H NMR of methyl (*trans*-4-hydroxycyclohexyl)acetate (methyl cyclohexylacetate). The orientation of H4 and H1 hydrogens was assigned as axial by using the axial-axial and axial-equatorial coupling constants of J_{H4} = 10.8, 4.1 Hz and J_{H1} = 3.8, 7.4 Hz.

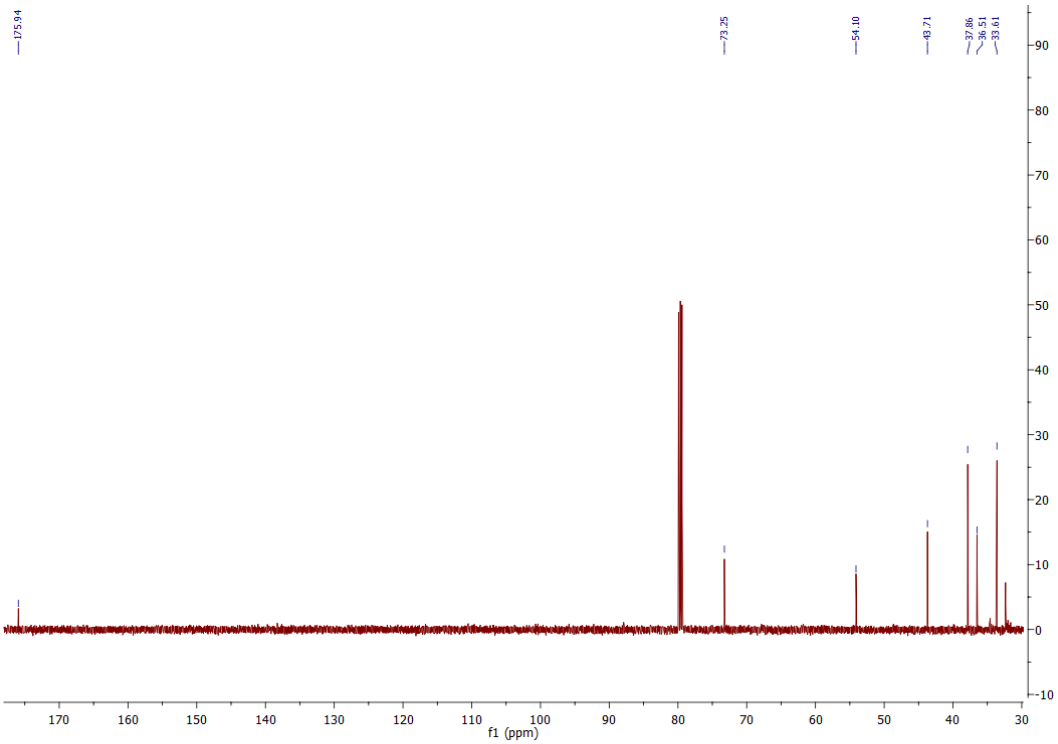


Figure S5 xl ^{13}C NMR of methyl (*trans*-4-hydroxycyclohexyl)acetate.

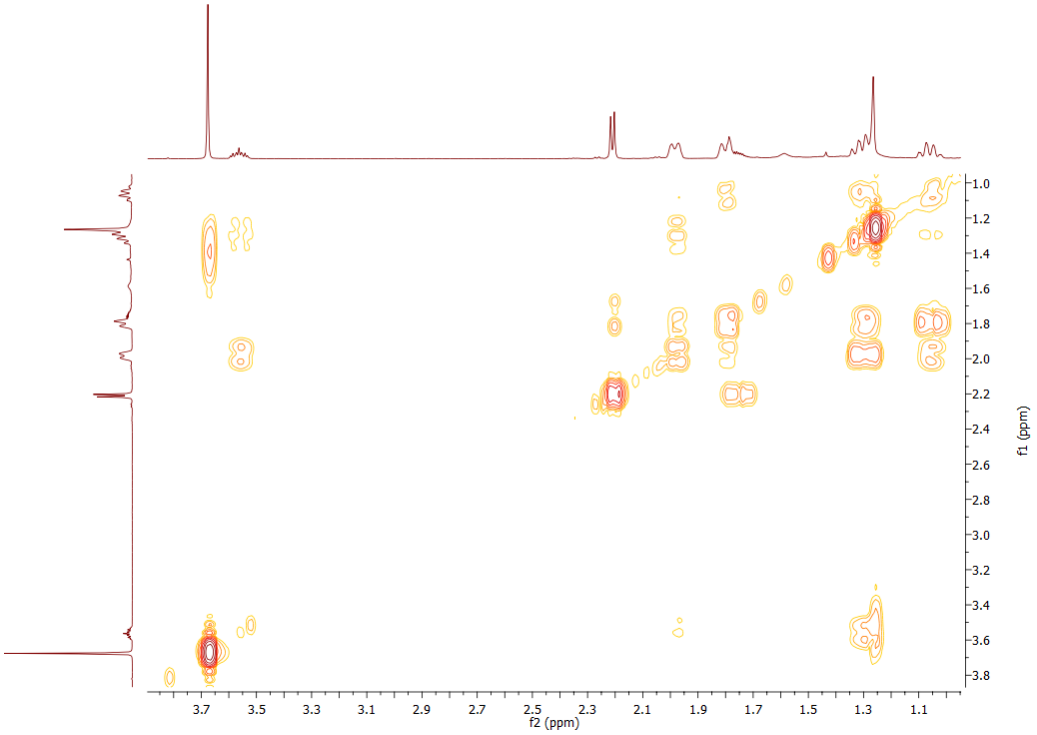


Figure S5 xli gCOSY NMR of methyl (*trans*-4-hydroxycyclohexyl)acetate.

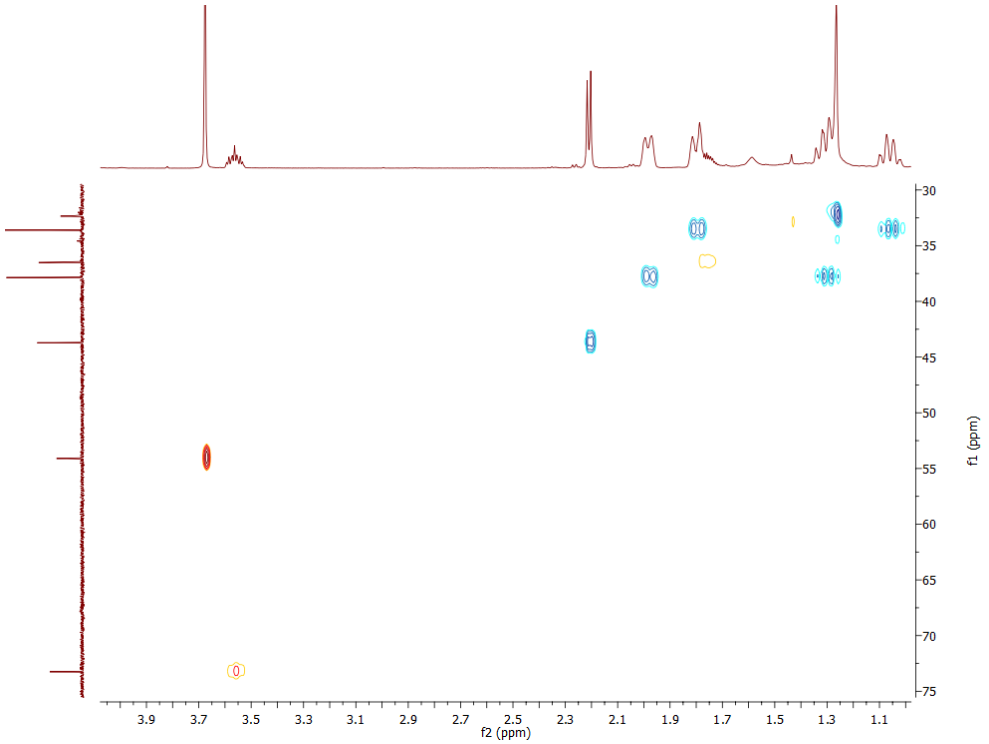


Figure S5 xlii HSQC NMR of methyl (*trans*-4-hydroxycyclohexyl)acetate.

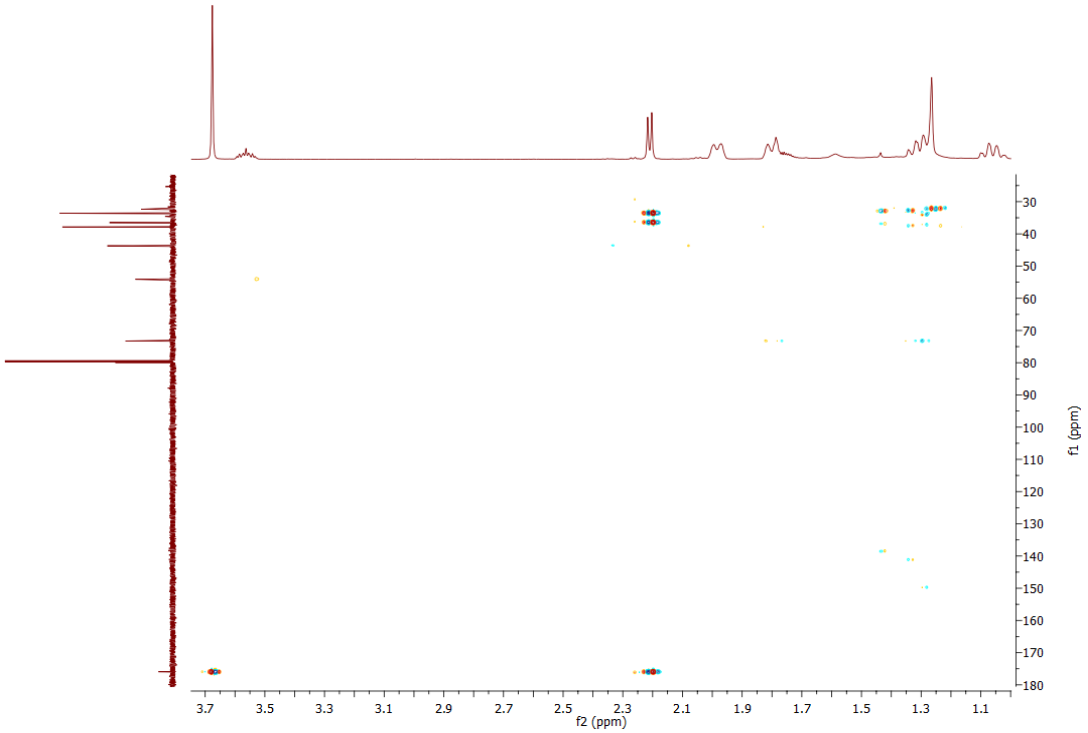


Figure S5 xliii HMBC NMR of methyl (*trans*-4-hydroxycyclohexyl)acetate.

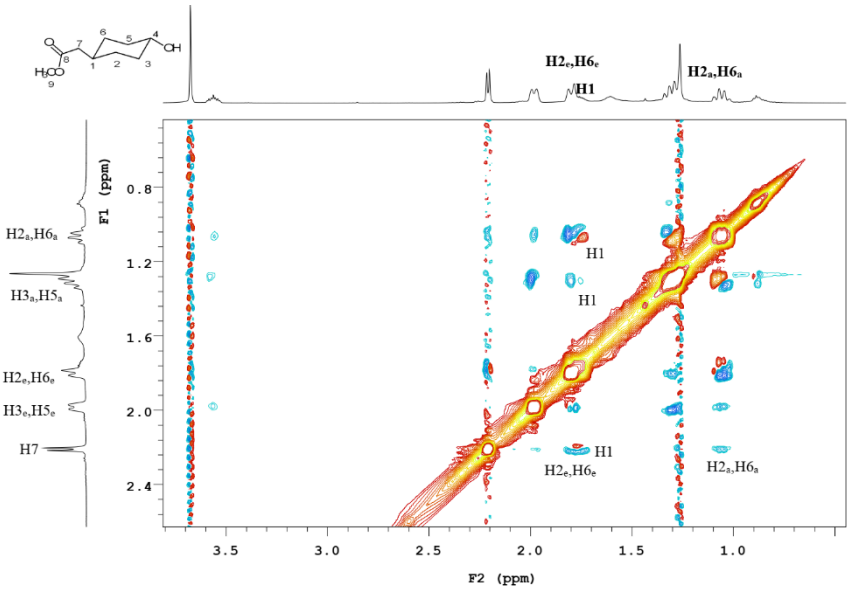


Figure S5 xliv Expansion of the ROESY NMR of methyl (*trans*-4-hydroxycyclohexyl)acetate which highlighted the interactions of H7 with the protons $H6_{ax/eq}$ of 1.12-1.00 ppm region.

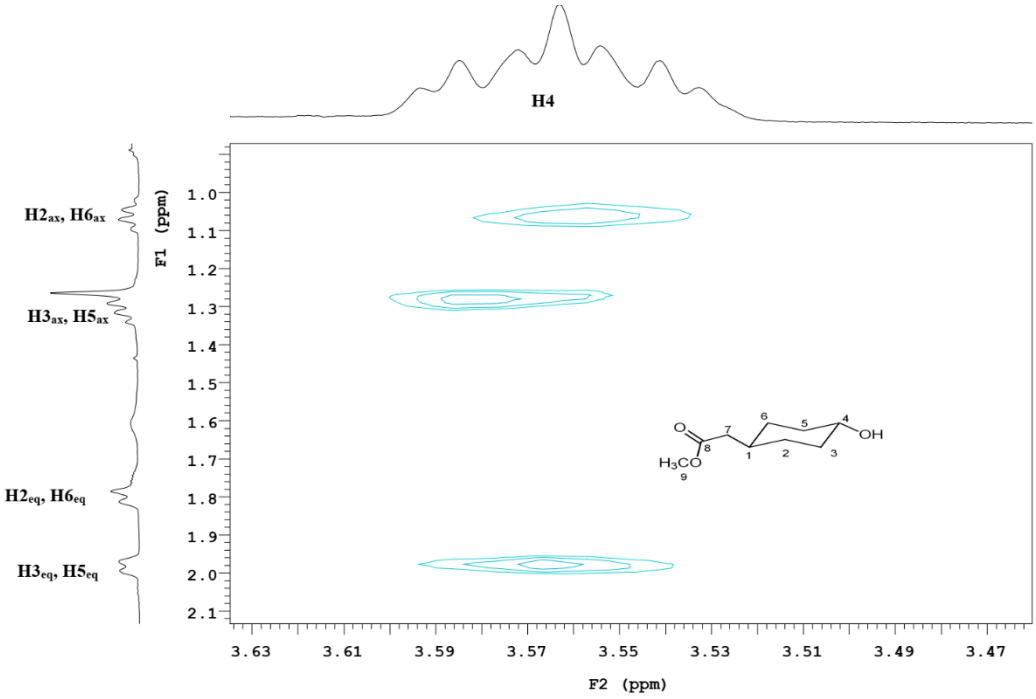


Figure 45 S5 xlv Expansion of the ROESY NMR of methyl (*trans*-4-hydroxycyclohexyl)acetate which highlighted the interactions of H4 (3.59-3.52 ppm) with the protons $H_{2/6ax}$ in 1.12-1.00 ppm region.

NMR data for ethyl (*trans*-4-hydroxycyclohexyl)acetate:

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.13 (*q*, *J* = 7.1 Hz, 2H, 2xH9), 3.59-3.50 (m, 1H, H4), 2.19 (d, *J* = 6.7 Hz, 2H, 2xH7), 2.02-1.91 (m, 2H, $H3_{eq}$ & $H5_{eq}$), 1.84-1.71 (m, 3H, H1, $H2_{eq}$ & $H6_{eq}$), 1.3-1.23 (m, 5H, $H3_{ax}$, $H5_{ax}$ & 3xH10), 1.11-1.01 (m, 2H, $H2_{ax}$ & $H6_{ax}$).

^{13}C NMR (126 MHz, CDCl3) δ 175.52 (C8), 73.26 (C4), 62.86 (C9), 43.98 (C7), 37.87 (C3 & C5), 36.54 (C1), 33.59 (C2 & C6), 16.62 (C10).

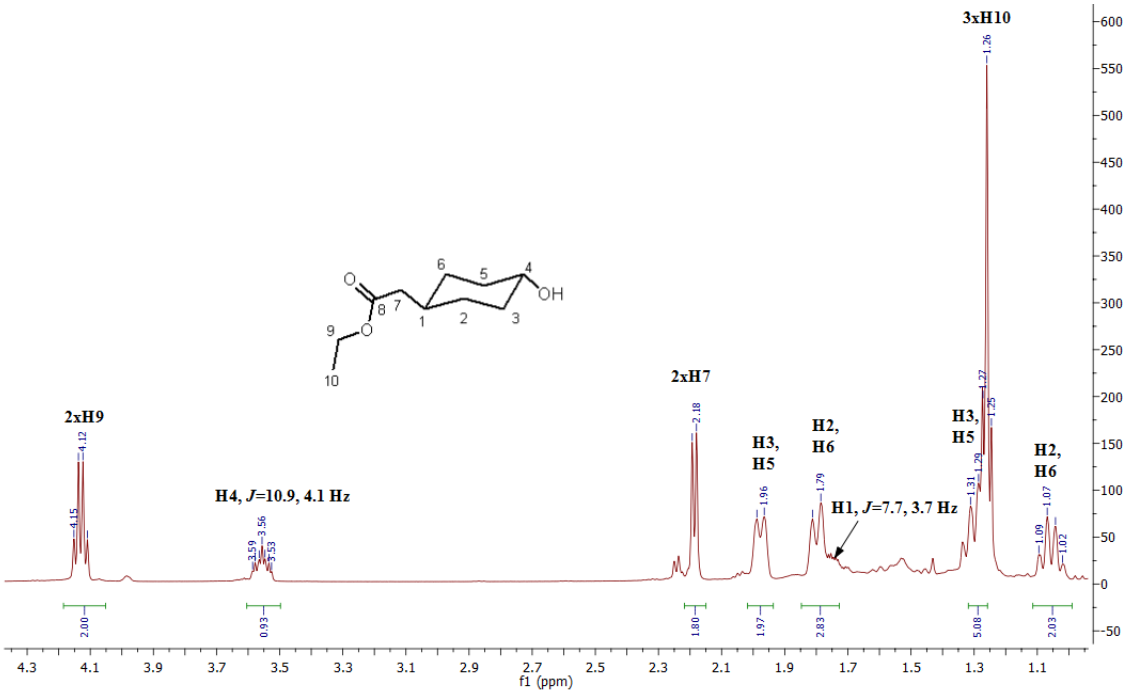


Figure S5 xlvi ^{1}H NMR of ethyl (*trans*-4-hydroxycyclohexyl)acetate (ethyl cyclohexylacetate). The orientation of H4 and H1 was assigned by the axial-axial and axial-equatorial coupling constants of J_{H4} = 10.9, 4.1 Hz and H1, J_{H1} = 7.7, 3.7 Hz.

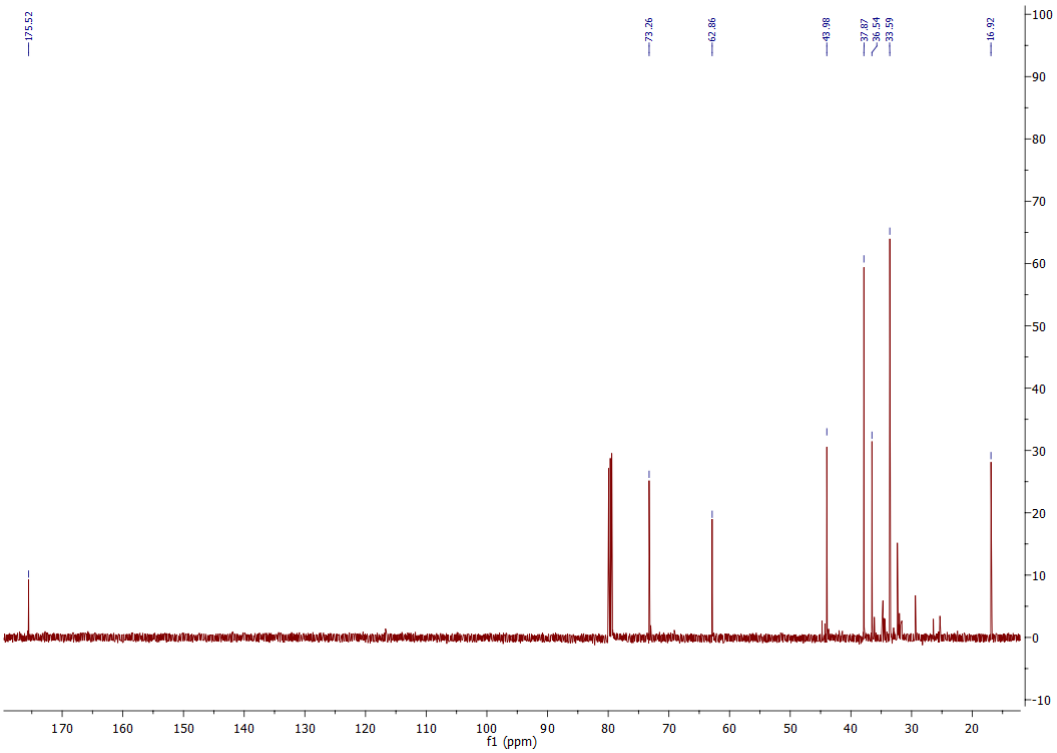


Figure S5 xlvii ^{13}C NMR of ethyl (*trans*-4-hydroxycyclohexyl)acetate.

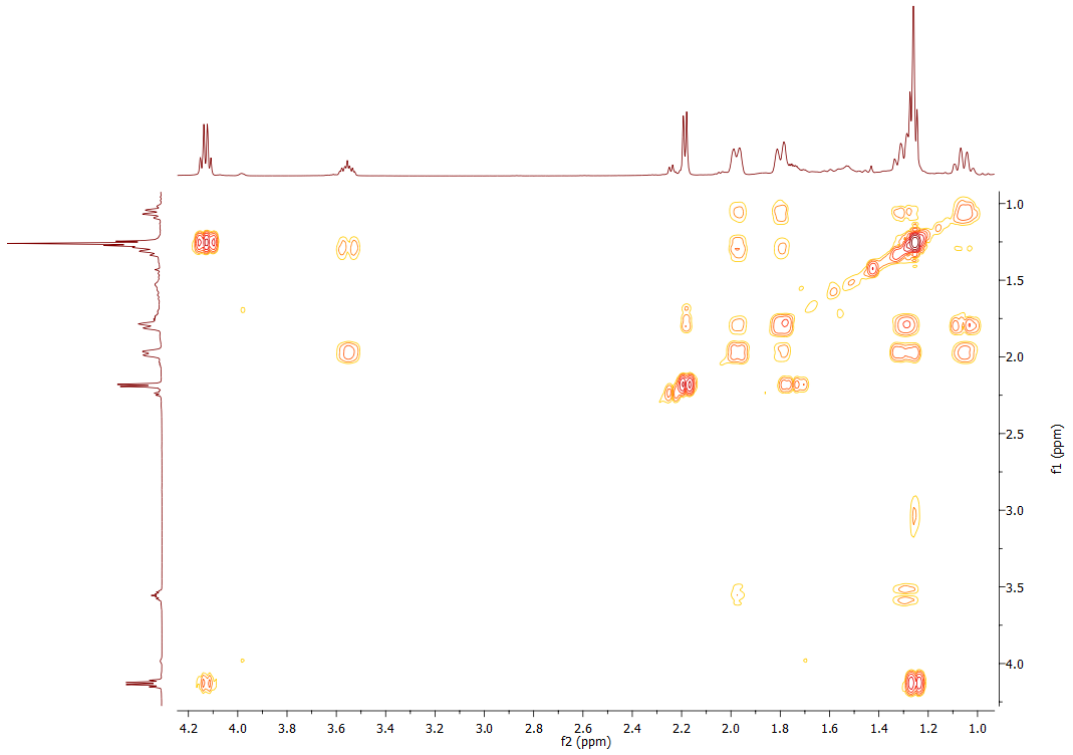


Figure S5 xlviii gCOSY NMR of ethyl (*trans*-4-hydroxycyclohexyl)acetate.

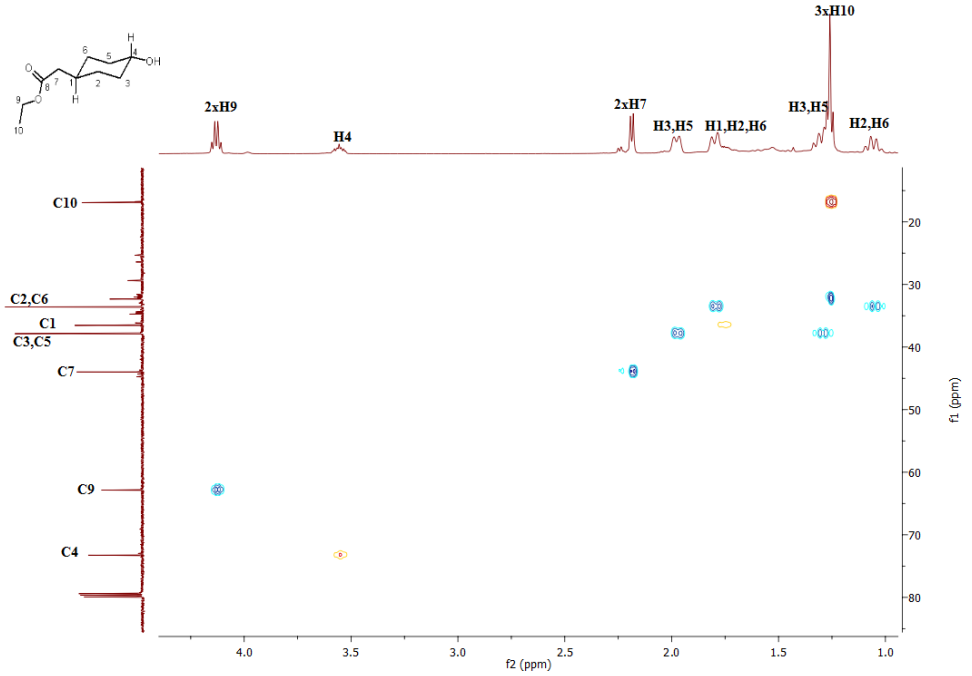


Figure S5 xlix HSQC NMR of ethyl (*trans*-4-hydroxycyclohexyl)acetate.

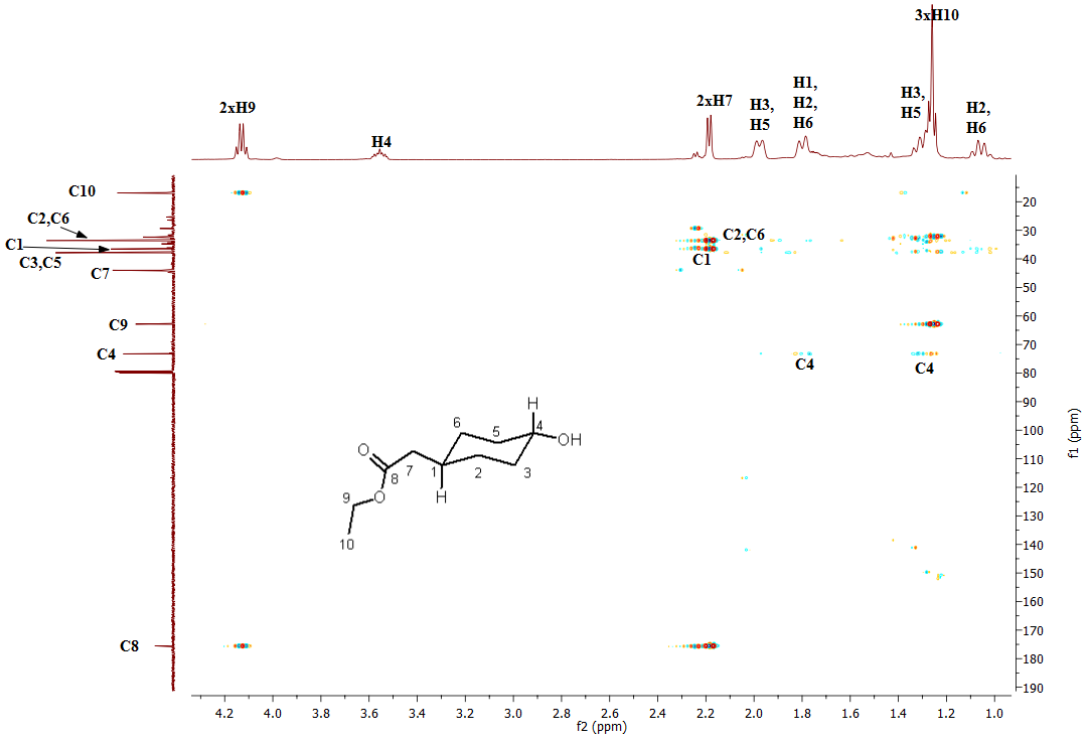


Figure S5 l HMBC NMR of ethyl (*trans*-4-hydroxycyclohexyl)acetate.

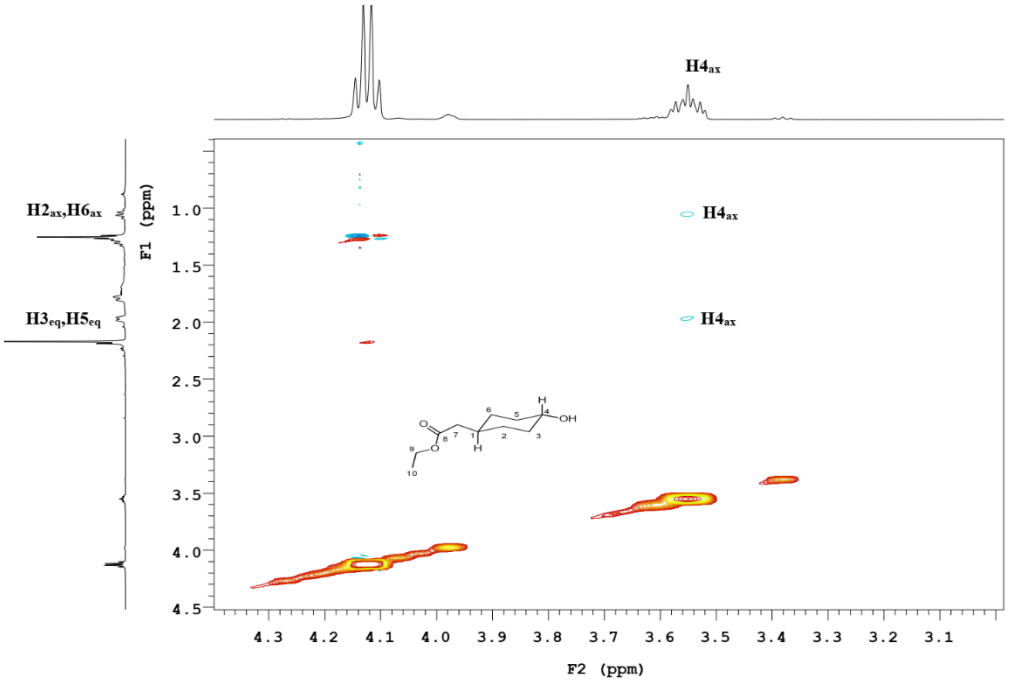


Figure S5 li Expansion of the ROESY NMR of ethyl (*trans*-4-hydroxycyclohexyl)acetate which highlighted the interactions of H4 (3.59-3.50 ppm) with the protons $H_{2/6ax}$ of 1.11-1.01 ppm region.

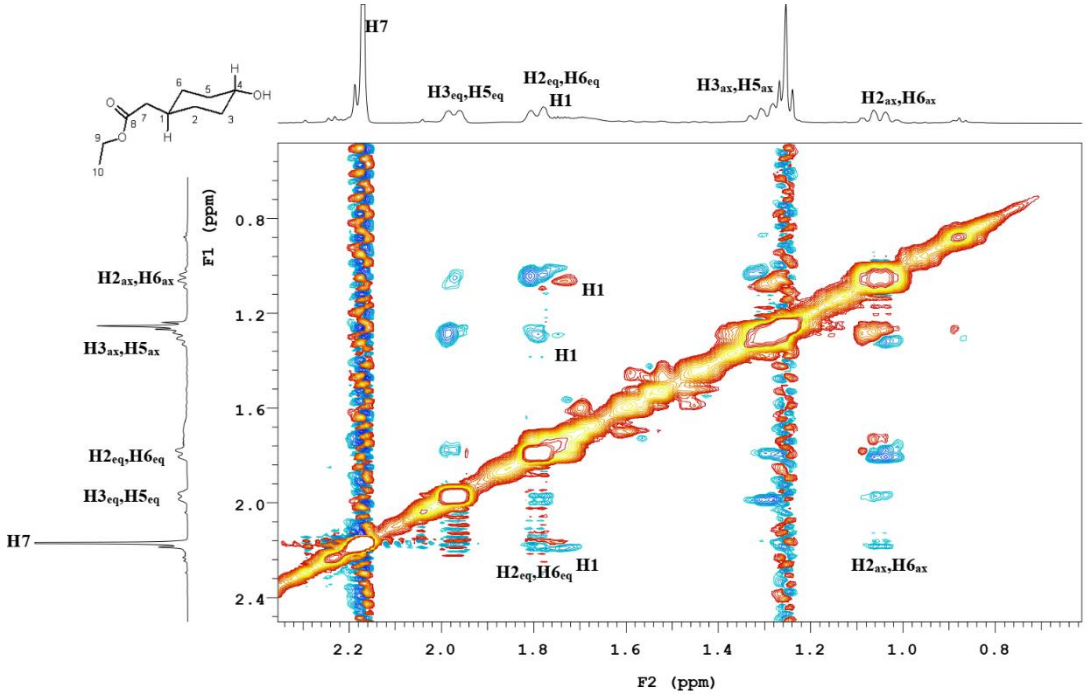
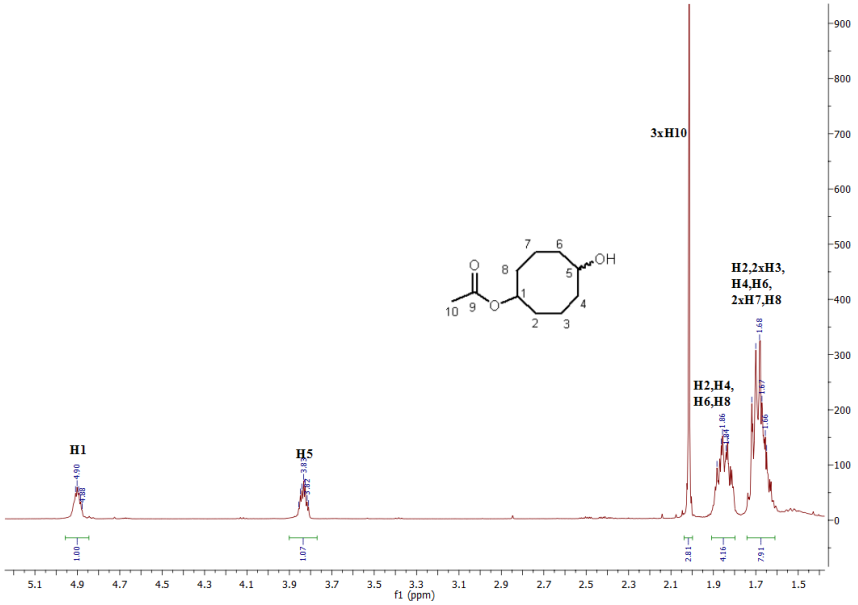


Figure S5 lii Expansion of the ROESY NMR of ethyl (*trans*-4-hydroxycyclohexyl)acetate which displayed the interactions of H7 (1.84-1.71 ppm) with the protons $H6_{ax/eq}$ of 1.11-1.01 ppm region.

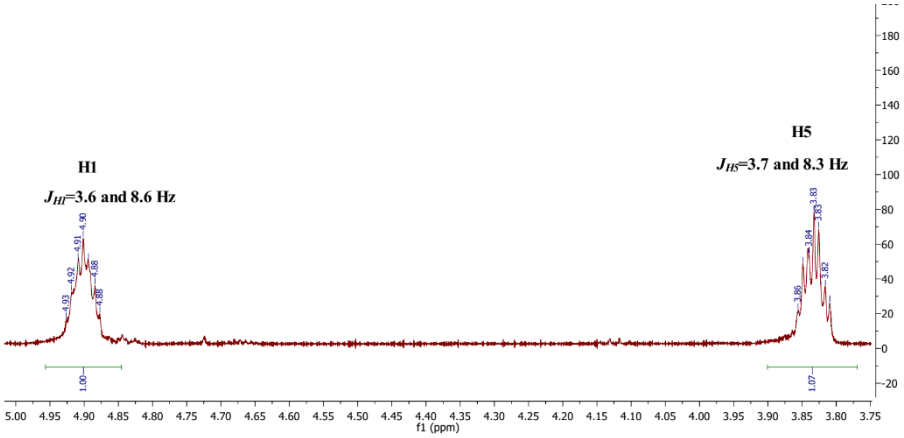
NMR for 5-hydroxycyclooctyl acetate:

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.93-4.85 (m, 1H, H1), 3.87-3.78 (m, 1H, H5), 2.01 (s, 3H, 3xH10), 1.90-1.77 (m, 4H, H2, H4, H6 & H8), 1.75-1.59 (m, 8H, H2, 2xH3, H4, H6, 2xH7 & H8).

^{13}C NMR (126 MHz, $CDCl_3$) δ 173.0 (C9), 76.61 (C1), 73.88 (C5), 37.87 (C4 & C6), 34.93 (C2 & C8), 24.13 (C10), 21.55 (C3 & C7).



(a)



(b)

Figure S5 liii (a) ^{1}H NMR of 5-hydroxycyclooctyl acetate. **(b)** Expansion of the ^{1}H NMR to highlight the H1 and H5 peaks. The coupling constants of H1 (3.6 and 8.6 Hz) and H5 (3.7 and 8.3 Hz) indicated both were in a similar orientation (both assigned as pseudoaxial). The metabolite was assigned as the *trans* isomer.

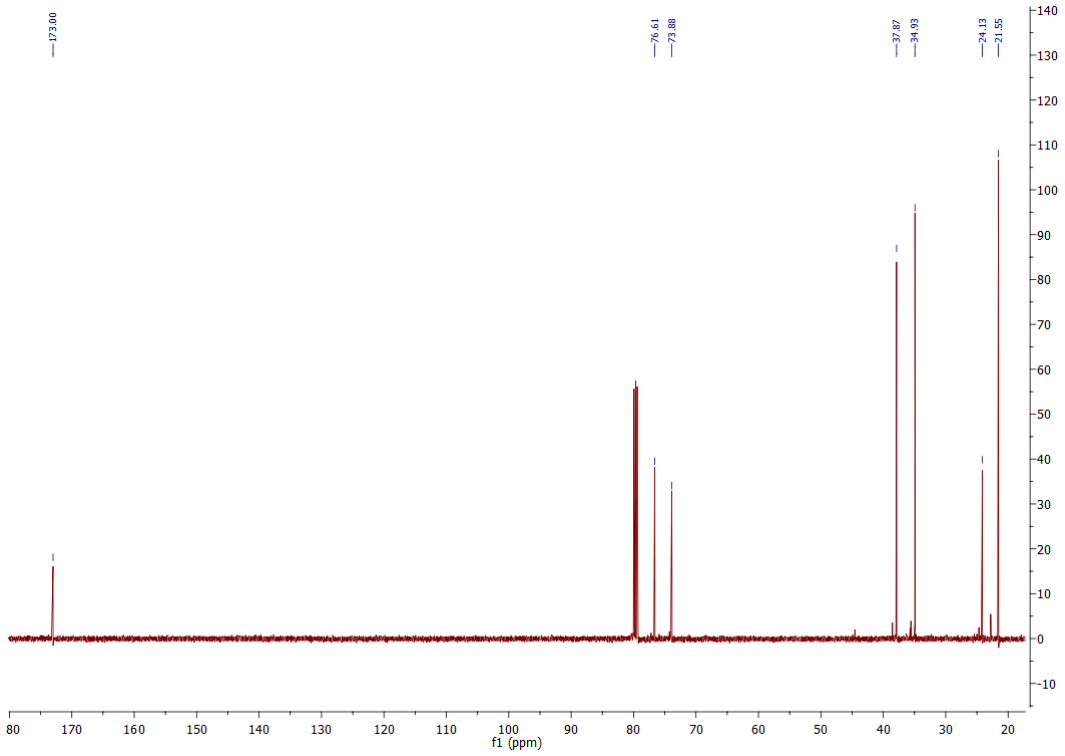


Figure S5 liv ^{13}C NMR of 5-hydroxycyclooctyl acetate. Minor signals indicated the presence of diastereomer of 5-hydroxycyclooctyl acetate (77.79, 74.25, 38.53, 35.58, 26.68, 22.79 ppm).

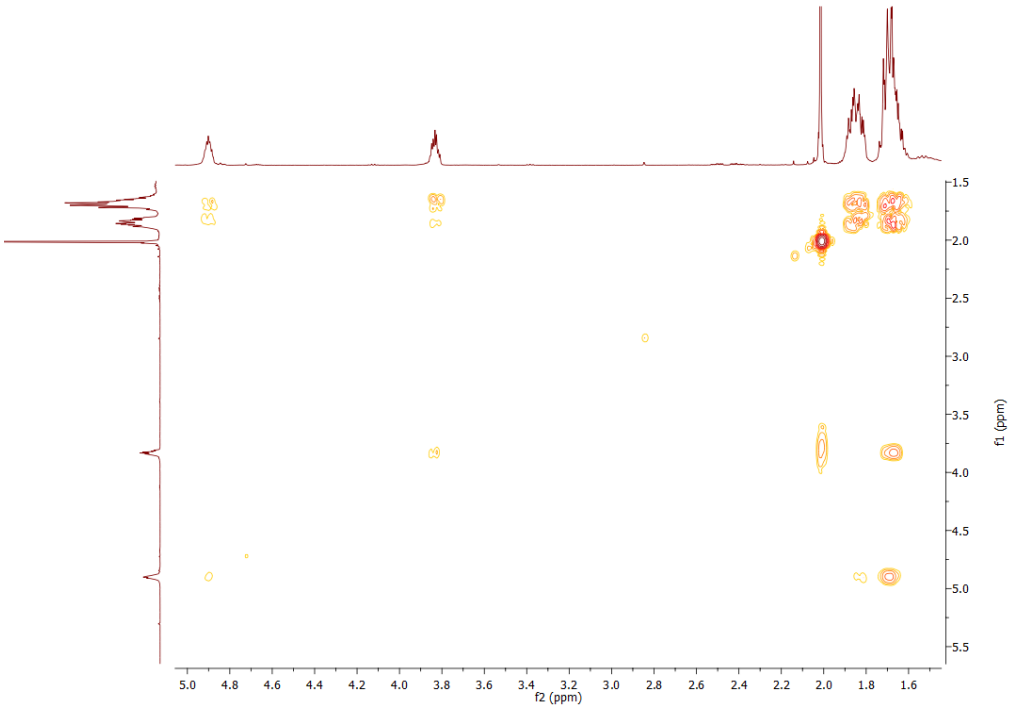


Figure S5 lv gCOSY NMR of 5-hydroxycyclooctyl acetate.

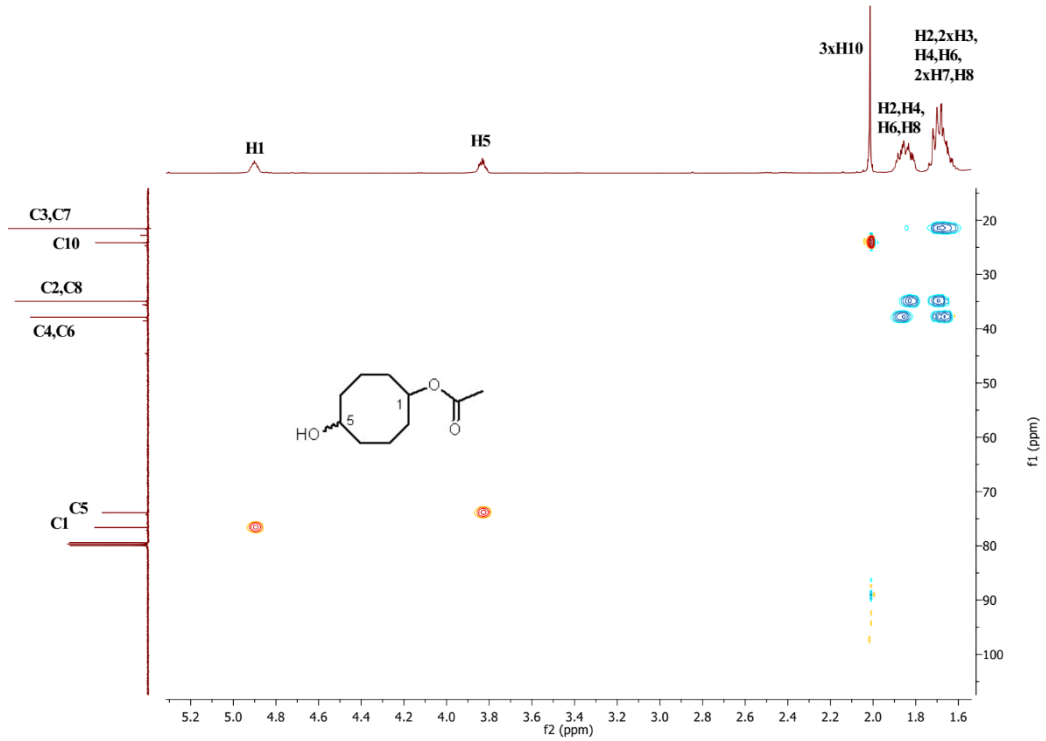


Figure S5 lvi HSQC NMR of 5-hydroxycyclooctyl acetate.

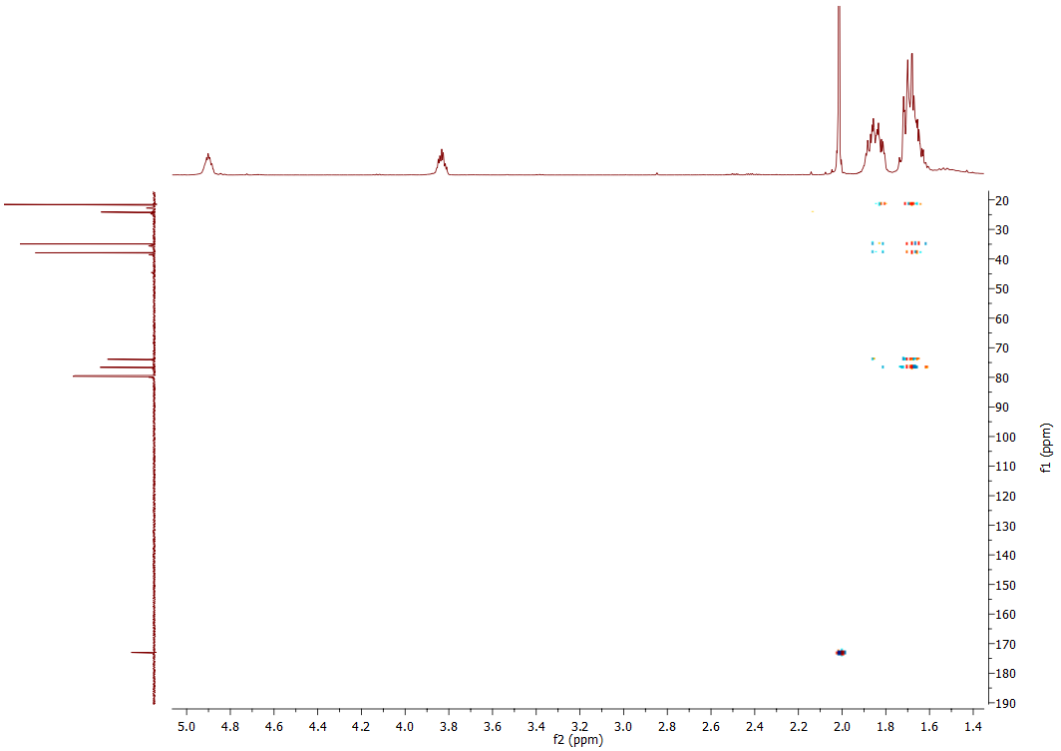
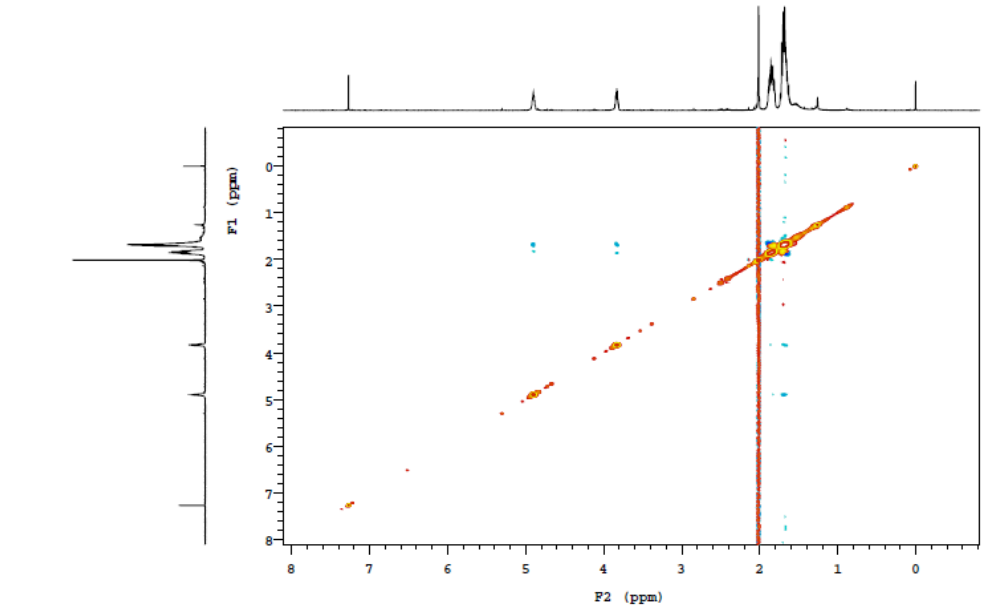
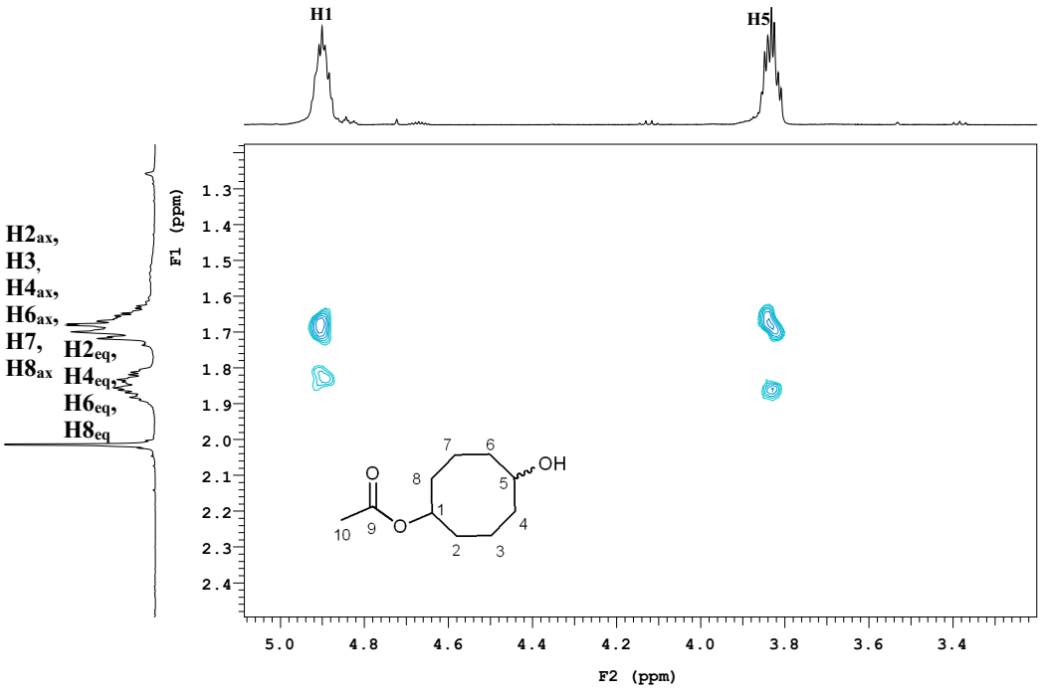


Figure S5 lvii HMBC NMR of 5-hydroxycyclooctyl acetate.



(a)



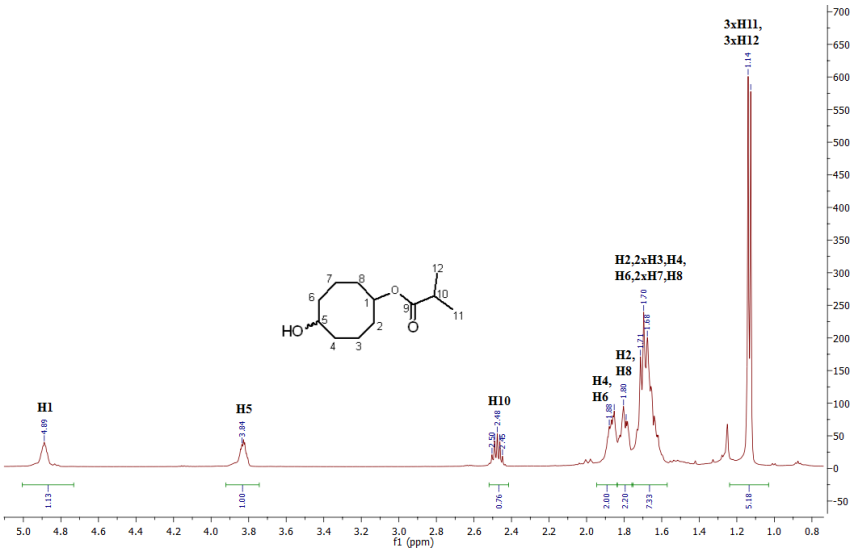
(b)

Figure S5 lviii (a) ROESY NMR of 5-hydroxycyclooctyl acetate. **(b)** Expansion of the ROESY NMR to highlight the strong interactions of H1 and H5 with the other protons.

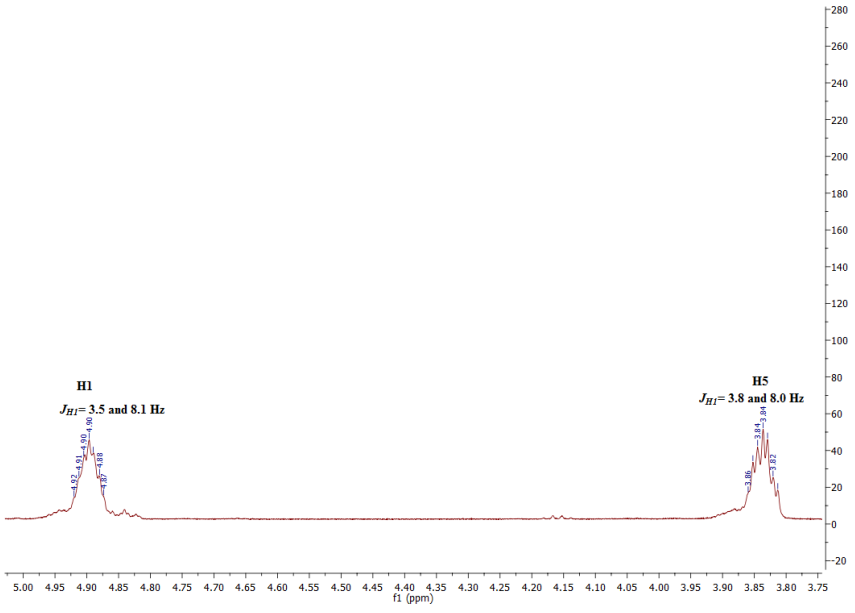
NMR for 5-hydroxycyclooctyl isobutyrate:

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.95-4.89 (m, 1H, H1), 3.88-3.74 (m, 1H, H5), 2.48 (*septet*, J = 7.0 Hz, 1H, H10), 1.94-1.88 (m, 2H, H4, H6), 1.82-1.76 (m, 2H, 2H, H2 & H8), 1.76-1.56 (m, 8H, H2, 2xH3, H4, H6, 2xH7 & H8), 1.13 (d, J = 7.0 Hz, 6H, 3xH11 & 3xH12).

^{13}C NMR (126 MHz, $CDCl_3$) δ 179.06 (C9), 76.10 (C1), 73.92 (C5), 37.85 (C4 & C6), 36.81 (C10), 34.92 (C2 & C8), 21.59 (C3, C7), 21.53 (C11 & C12).



(a)



(b)

Figure S5 lix (a) ^{1}H NMR of 5-hydroxycyclooctyl isobutyrate. **(b)** The coupling constants of H1 and H5 were similar to each other suggested their similar orientation (both pseudoaxial) therefore the metabolite proposed to be a *trans* isomer.

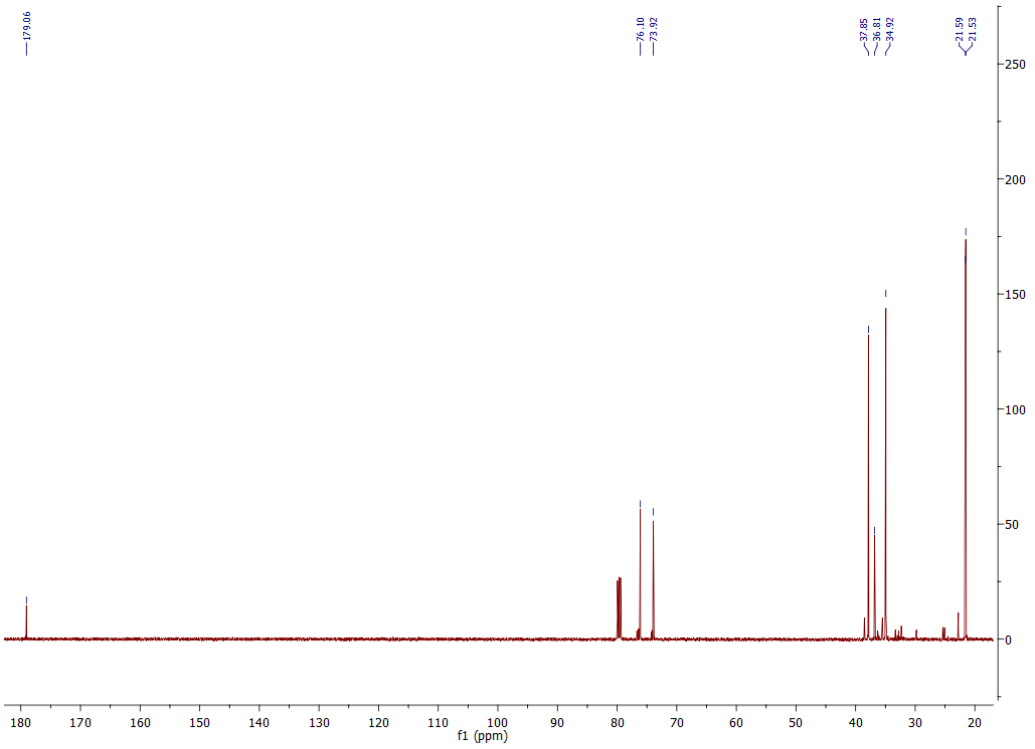


Figure S5 lx ^{13}C NMR of 5-hydroxycyclooctyl isobutyrate. Minor signals indicated the presence of other (*cis*) diastereomer of 5-hydroxycyclooctyl isobutyrate and a minor unidentified metabolite. The minor carbon signals of the *cis* isomer at both were found at: 76.78, 74.07, 38.53, 36.86 and 35.50 and showed similar relative intensity to the *trans* isomer. Full characterisation of these metabolites were not possible due to low yield and poor separation.

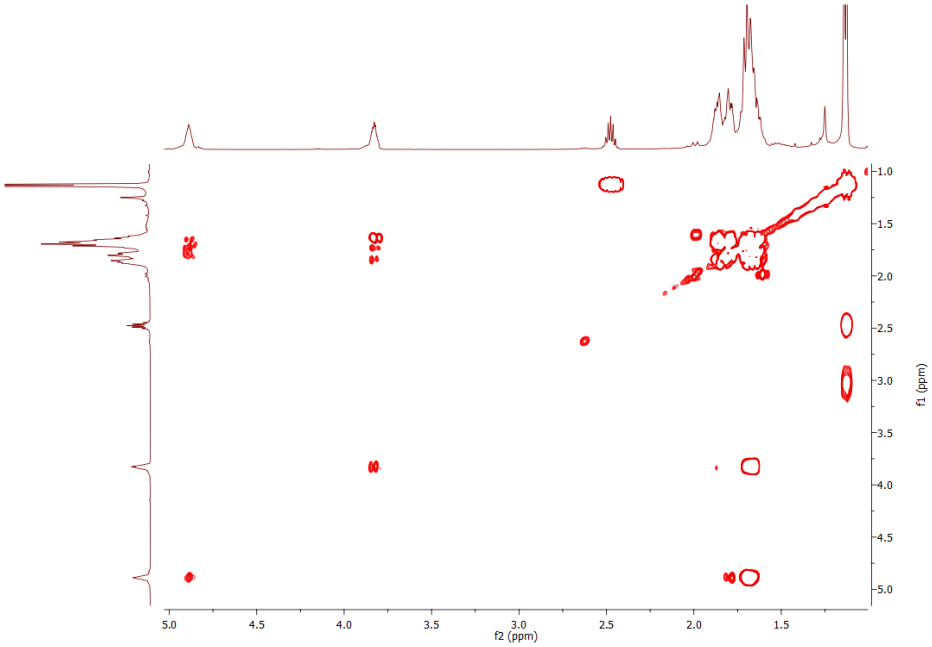


Figure S5 lxi gCOSY NMR of 5-hydroxycyclooctyl isobutyrate.

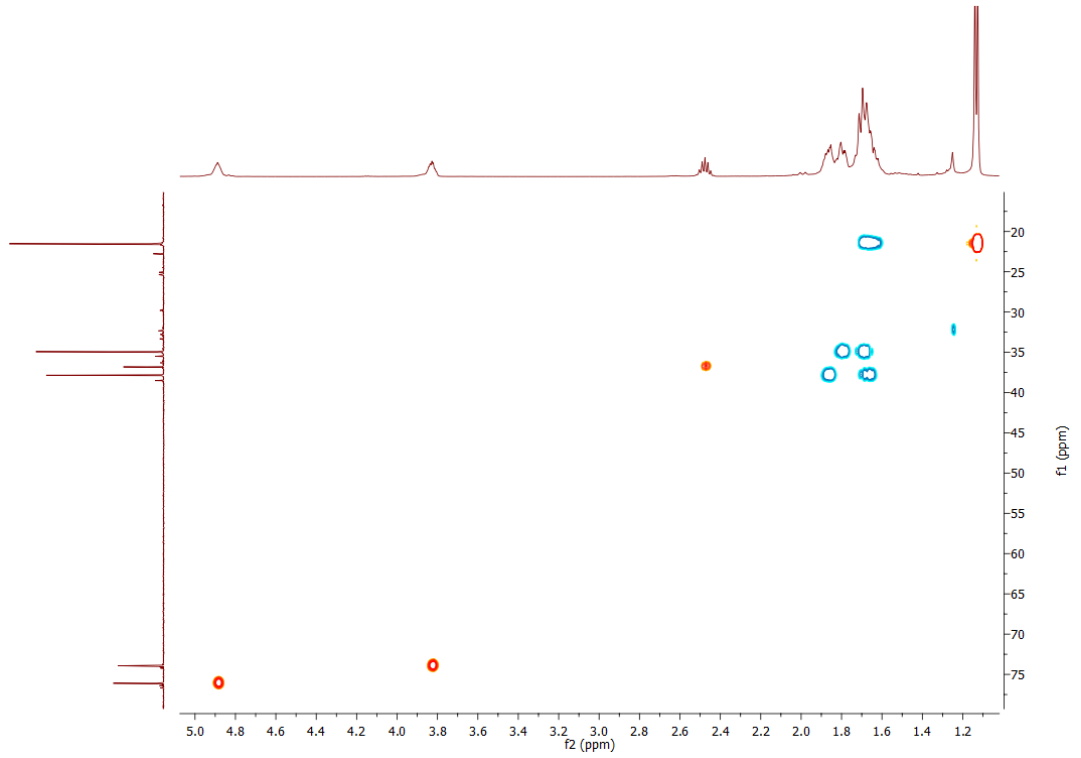


Figure S5 lxii HSQC NMR of 5-hydroxycyclooctyl isobutyrate.

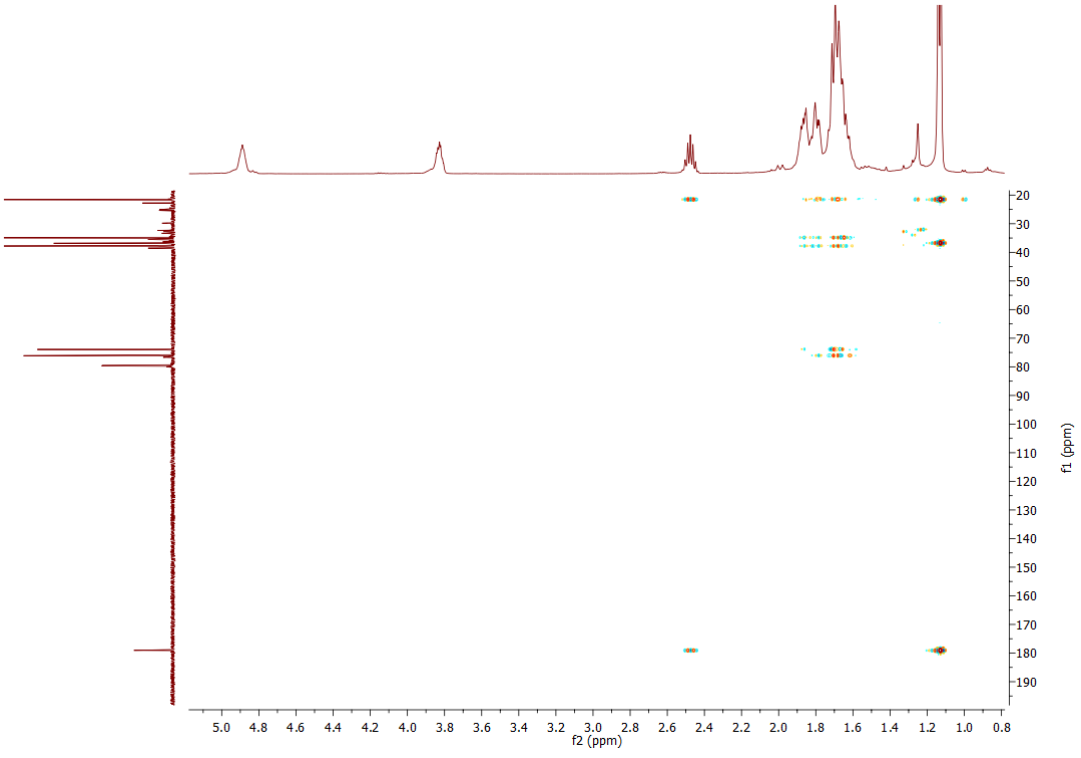
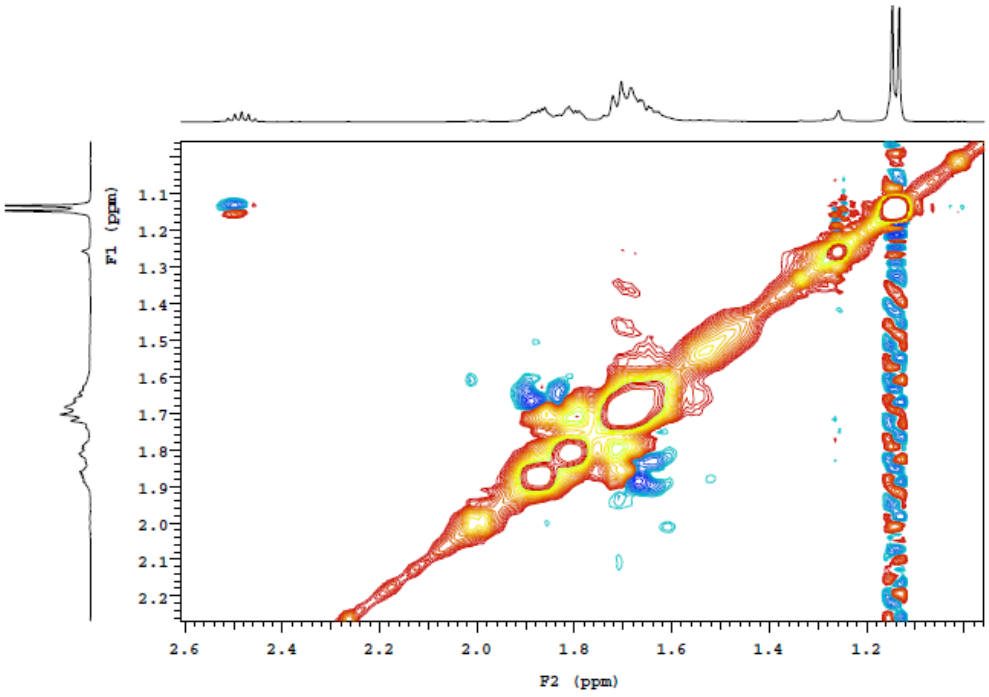
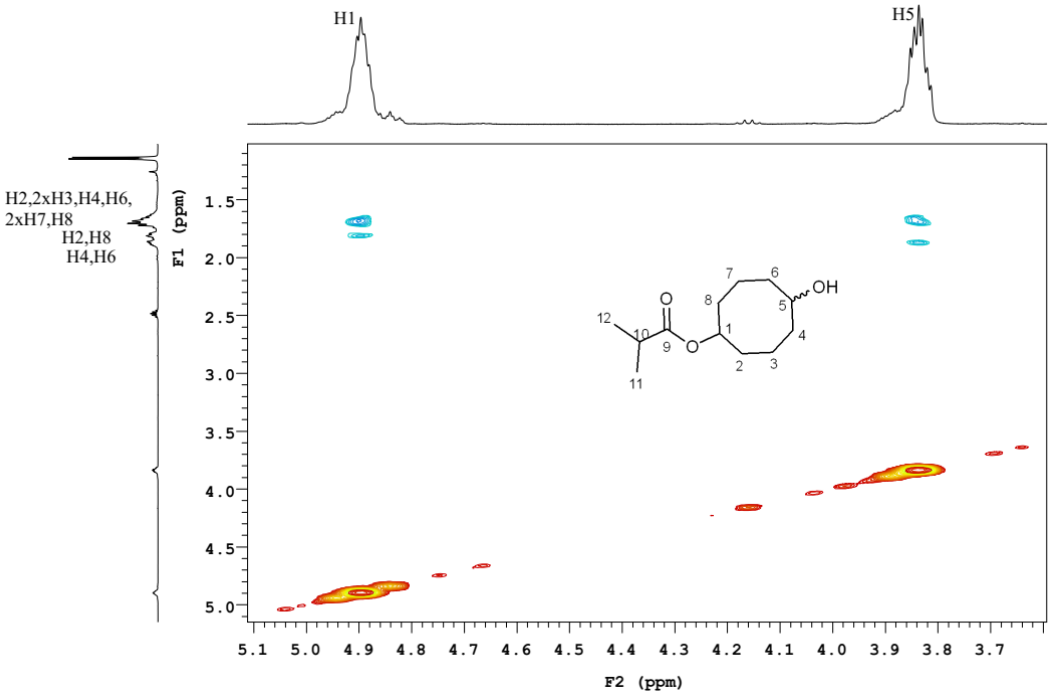


Figure S5 lxiii HMBC NMR of 5-hydroxycyclooctyl isobutyrate.



(a)



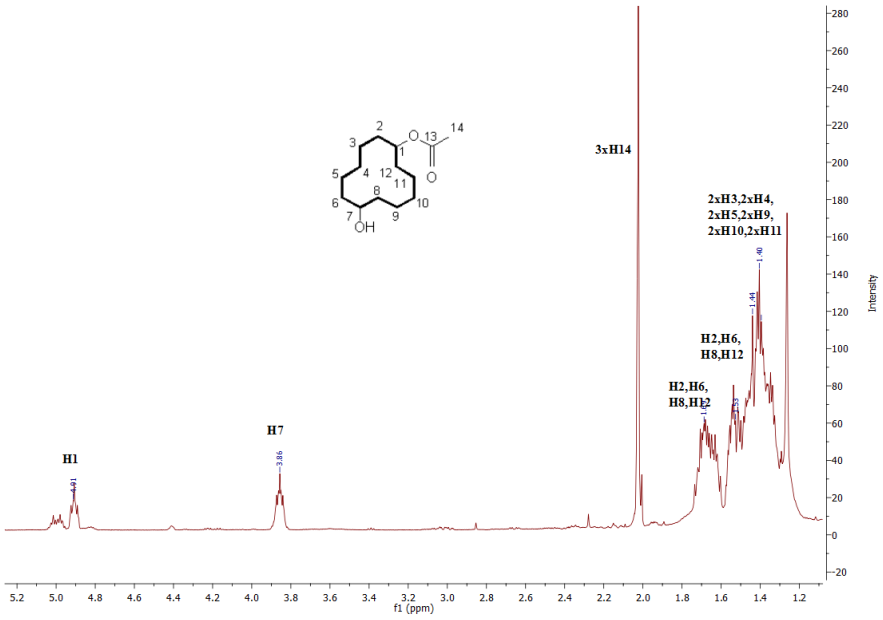
(b)

Figure S5 liv ROESY NMR of 5-hydroxycyclooctyl isobutyrate. **(b)** Expansion of the ROESY NMR to highlight the strong interactions of H1 and H5 with adjacent protons.

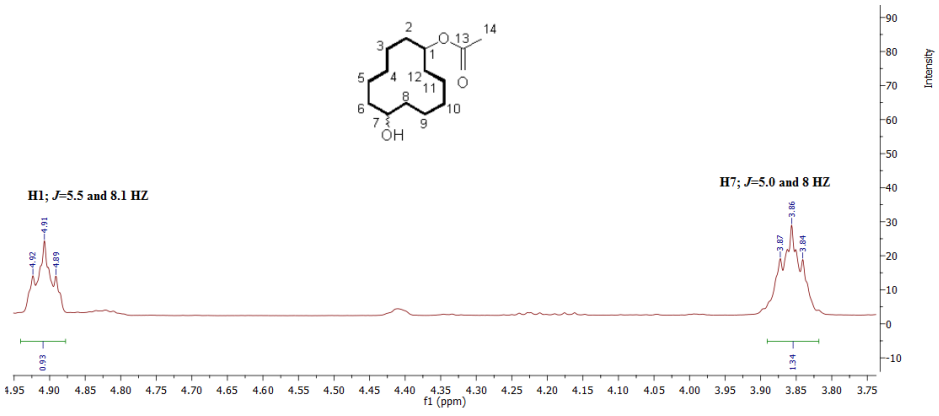
NMR for 7-hydroxycyclododecyl acetate [6]

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.91 (*t*, *J* = 8.1 Hz, 1H, H1), 3.89-3.80 (m, 1H, H7), 2.02 (s, 3H, 3xH14), 1.73-1.61 (m, 4H, H2, H6, H8 & H12), 1.57-1.50 (m, 4H, H2, H6, H8 & H12), 1.48-1.32 (m, 12H, 2xH3, 2xH4, 2xH5, 2xH9, 2xH10 & 2xH11).

^{13}C NMR (126 MHz, $CDCl_3$) δ 170.57 (C13), 73.61 (C1), 70.83 (C7), 30.83 (C2 & C12), 27.53 (C6 & C8), 25.88 (C3 & C11), 21.36 (C14), 19.14 (C5 & C9), 18.97 (C4 & C10).

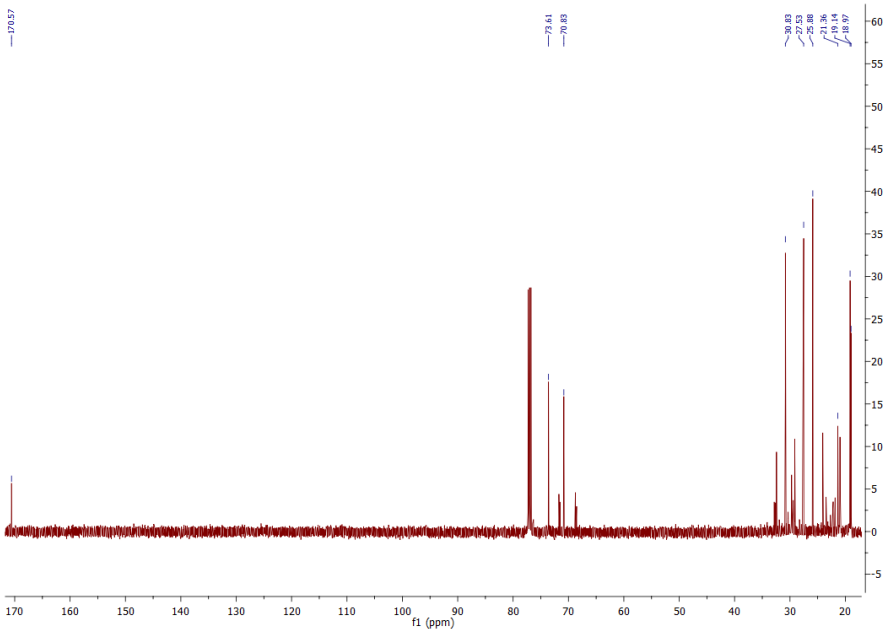


(a)

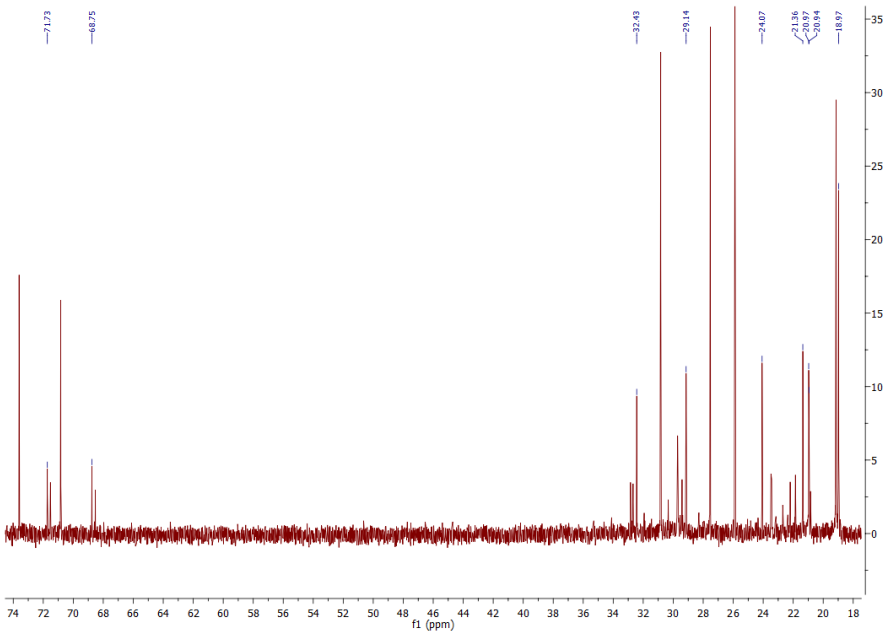


(b)

Figure S5 lxiv (a) ^{1}H NMR of 7-hydroxycyclododecyl acetate [6]. **(b)** Expansion of the proton NMR to highlight the H1 and H7 peaks. The H1 and H7 peaks and their coupling constants J_{H1} = 5.5, 8.1 Hz and J_{H7} = 5.0, 8.0 Hz are almost identical suggested both protons are in the same orientation (both axial). The product therefore assigned as a *trans* isomer.



(a)



(b)

Figure S5 lxvi (a) ^{13}C NMR of 7-hydroxycyclododecyl acetate (*trans*) [6]. The product was assigned as 7-hydroxy as the ^{13}C NMR showed symmetrical carbon peaks in the cyclododecyl ring. **(b)** The NMR highlighted the minor carbon signals of the other diastereomer of 7-hydroxycyclododecyl acetate (*cis*) [6]. The ^{13}C NMR also had additional minor product signals, that product was proposed diastereomer of 5-hydroxycyclododecyl acetate (see below).

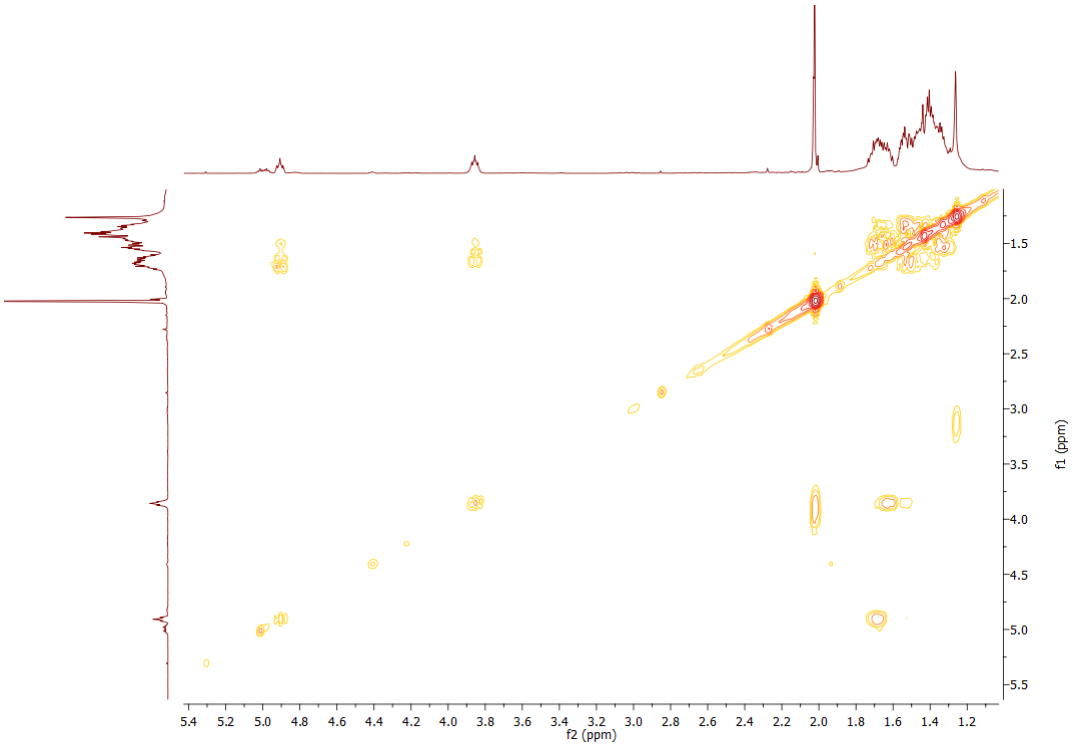
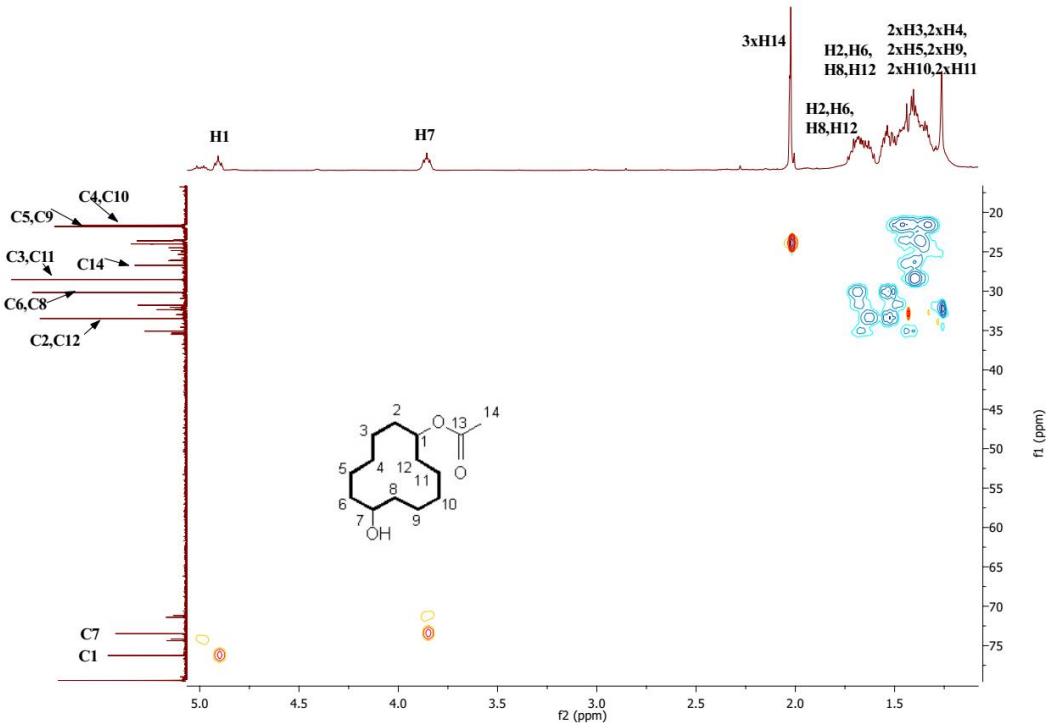
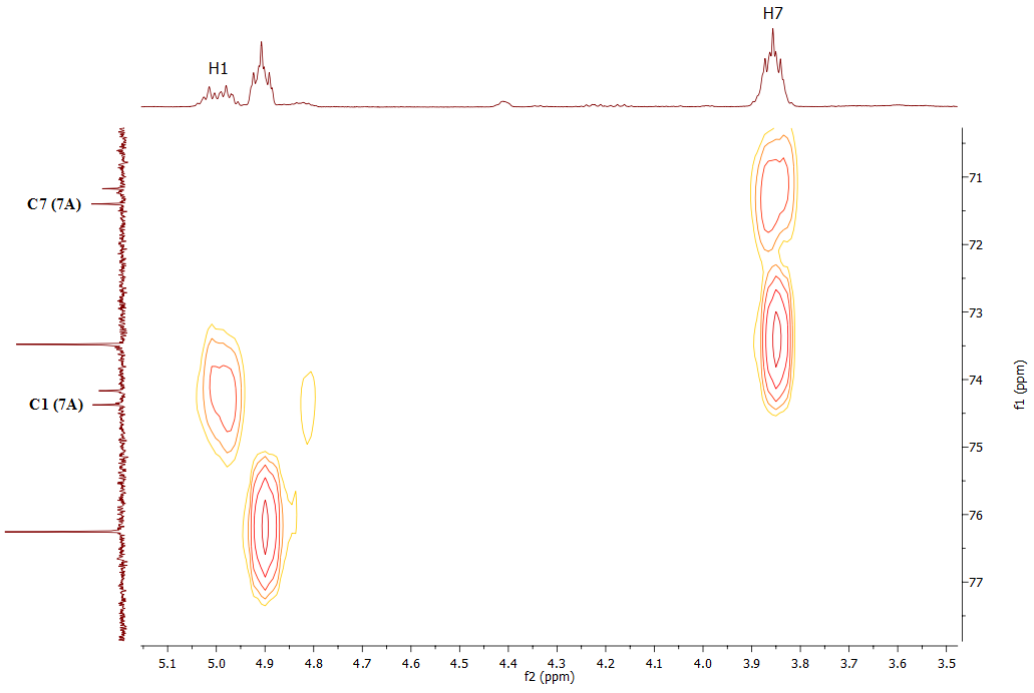


Figure S5 lxvii gCOSY NMR of 7-hydroxycyclododecyl acetate.



(a)



(b)

Figure S5 lxviii (a) HSQC NMR of 7-hydroxycyclododecyl acetate (*trans*). **(b)** Expansion of the HSQC NMR in 65 to 80 ppm region to highlight the interactions of minor products C1 and C7 with the proton of 5.04-4.99 ppm and 3.9-3.81 ppm regions. C1 and C7 (A): 7-hydroxy cyclododecyl acetate (*cis*) [6].

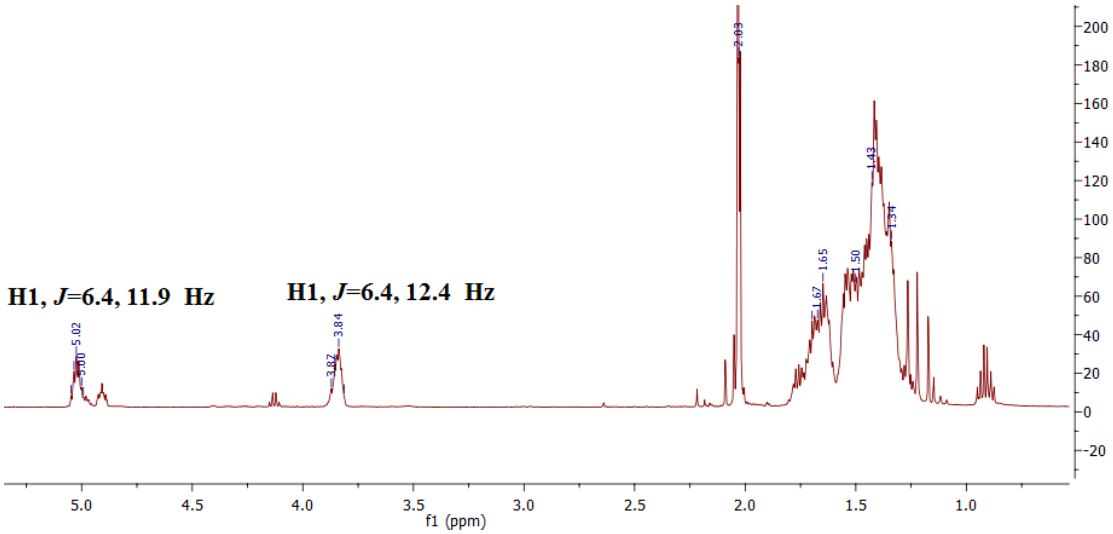


Figure 69 ^{1}H NMR of 5-hydroxycyclododecyl acetate.

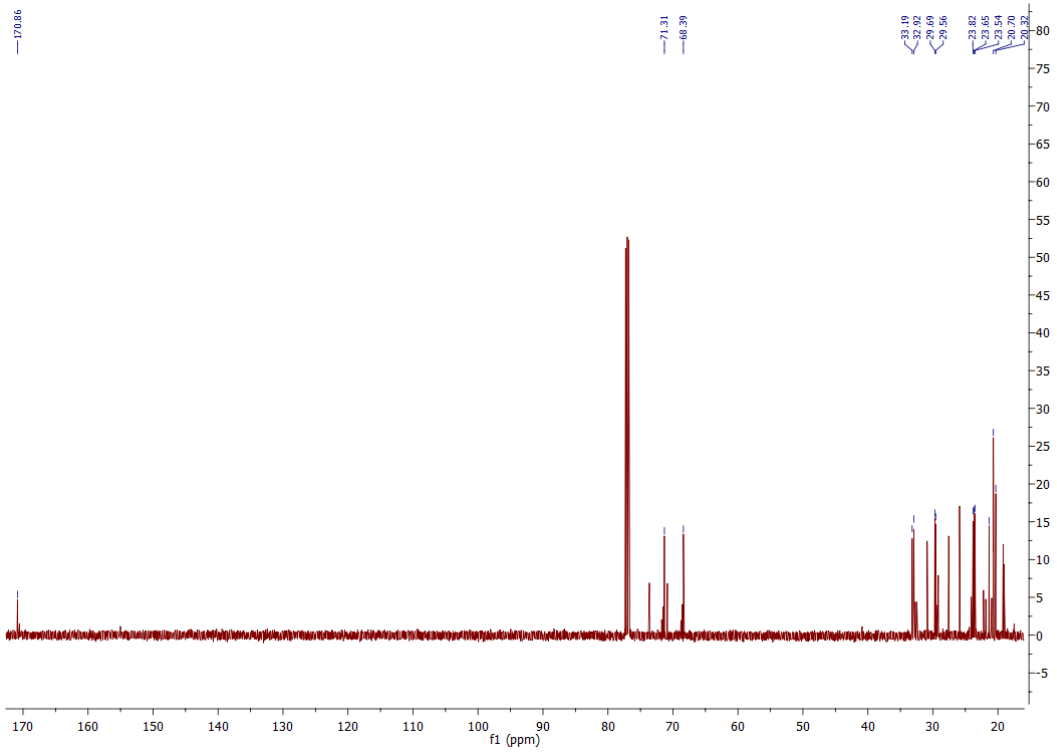
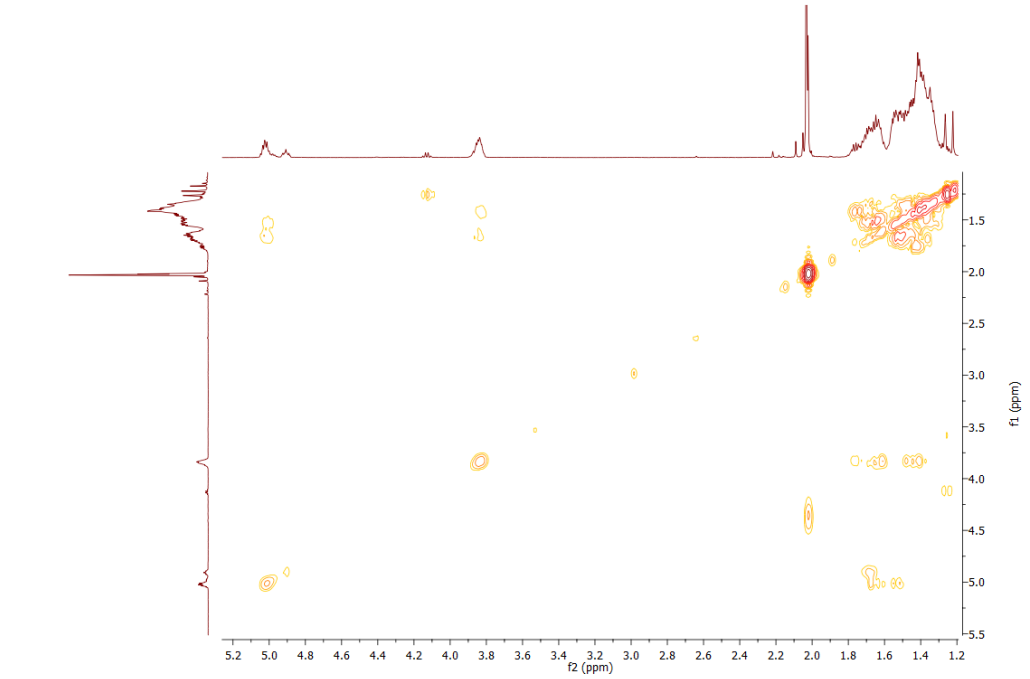
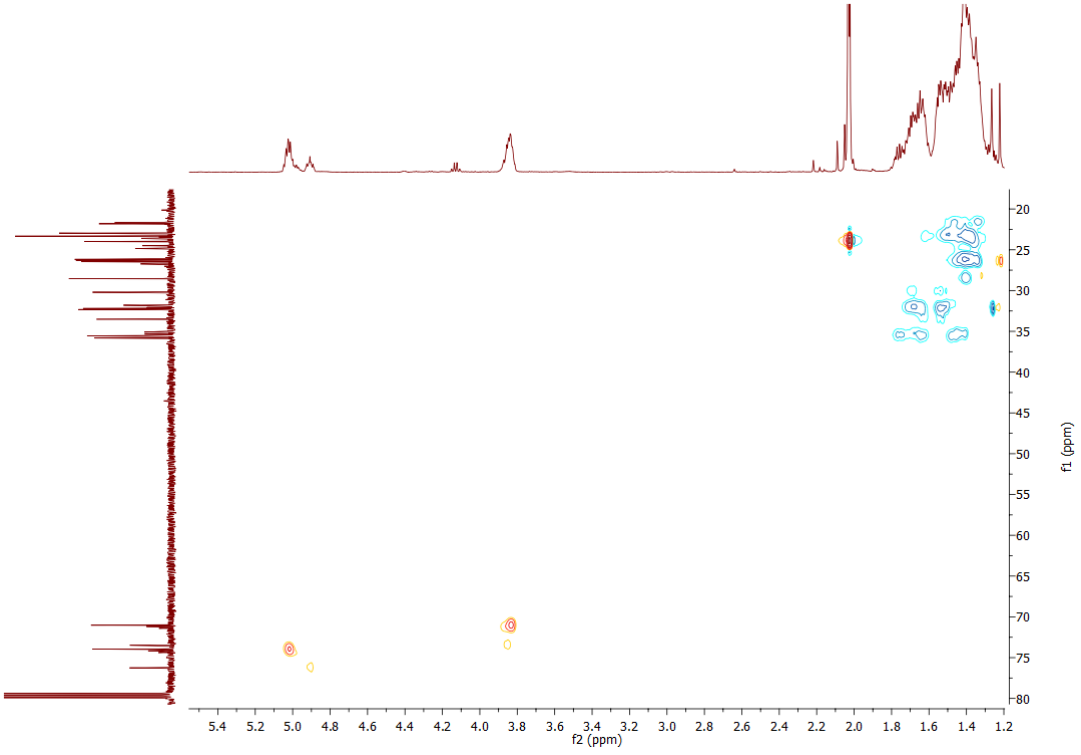


Figure S5 lxix ^{13}C NMR highlighted the carbon signals of 5-hydroxycyclododecyl acetate.



(a)



(b)

Figure S5 lxx (a) gCOSY NMR of 5-hydroxycyclododecyl acetate. **(b)** HSQC NMR of 5-hydroxycyclododecyl acetate.

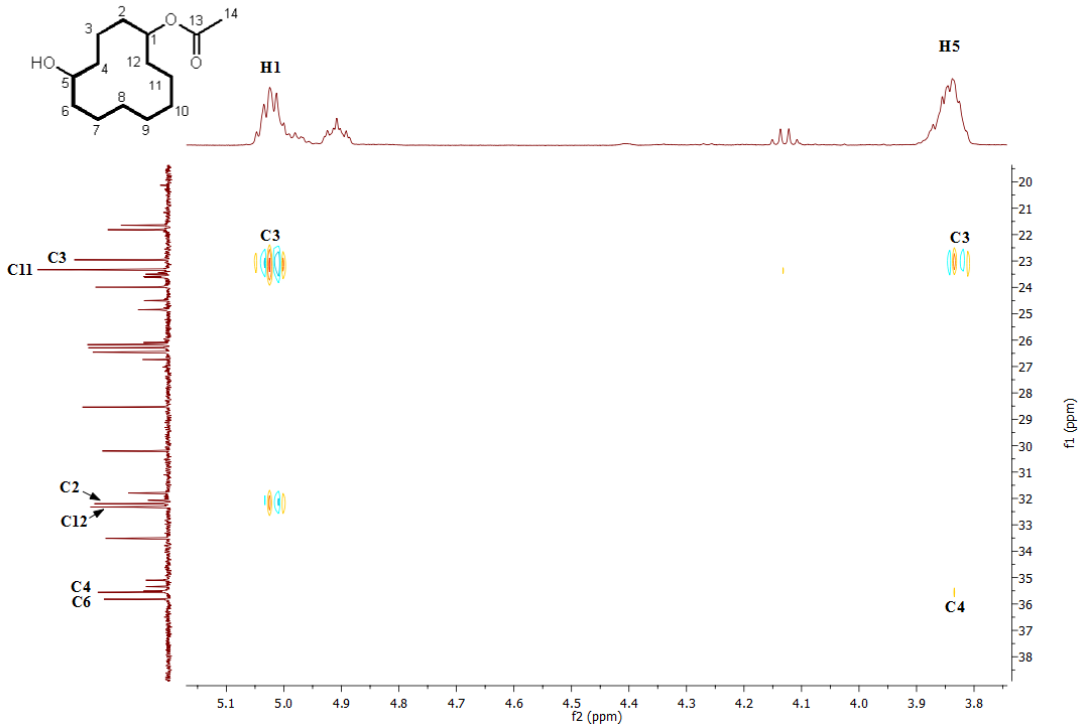


Figure S5 lxxi HMBC NMR of 5-hydroxycyclododecyl acetate. The metabolite was confirmed 5-hydroxy metabolite as both H1 and H5 showed strong interactions with C3.

Table S3 ^{13}C NMR signals of the different products observed in the NMR of metabolites of cyclodocecyl acetate (See Figure S3).

Products	**^{13}C NMR signals**
5-hydroxycyclododecyl acetate	170.86, 71.31, 68.39, 33.19, 32.92, 30.88 29.69, 29.56, 23.82, 23.63, 23.54, 21.36, 20.70, 20.32
Unidentified metabolite: another alcohol or the other diastereomer of 5-hydroxycyclododecyl acetate, Signals observed in ^{13}C NMR of 5-hydroxycyclododecyl acetate	71.54, 68.55, 32.88, 32.71, 29.44, 23.51, 23.46, 22.22, 21.87, 21.36, 20.99, 20.96, 20.87, C=O peak not detected
trans-7-hydroxycyclododecyl acetate [6]	170.57, 73.61, 70.83, 30.83, 27.53, 25.88, 21.36, 19.14, 18.97
cis-7-hydroxycyclododecyl acetate [6]	71.73, 68.75, 32.43, 29.14, 24.07, 21.36, 20.97, 20.94, 18.97, C=O peak not detected

Figure S6 NMR data for the ester substrates

MR data for cyclohexyl isobutyrate:

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.82-4.66 (m, 1H, H1), 2.52 (*septet*, J = 7.0 Hz, 1H, H8), 1.88-1.66 (m, 10H), 1.16 (d, J = 7.0 Hz, 6H, 3xH9 & 3xH10).

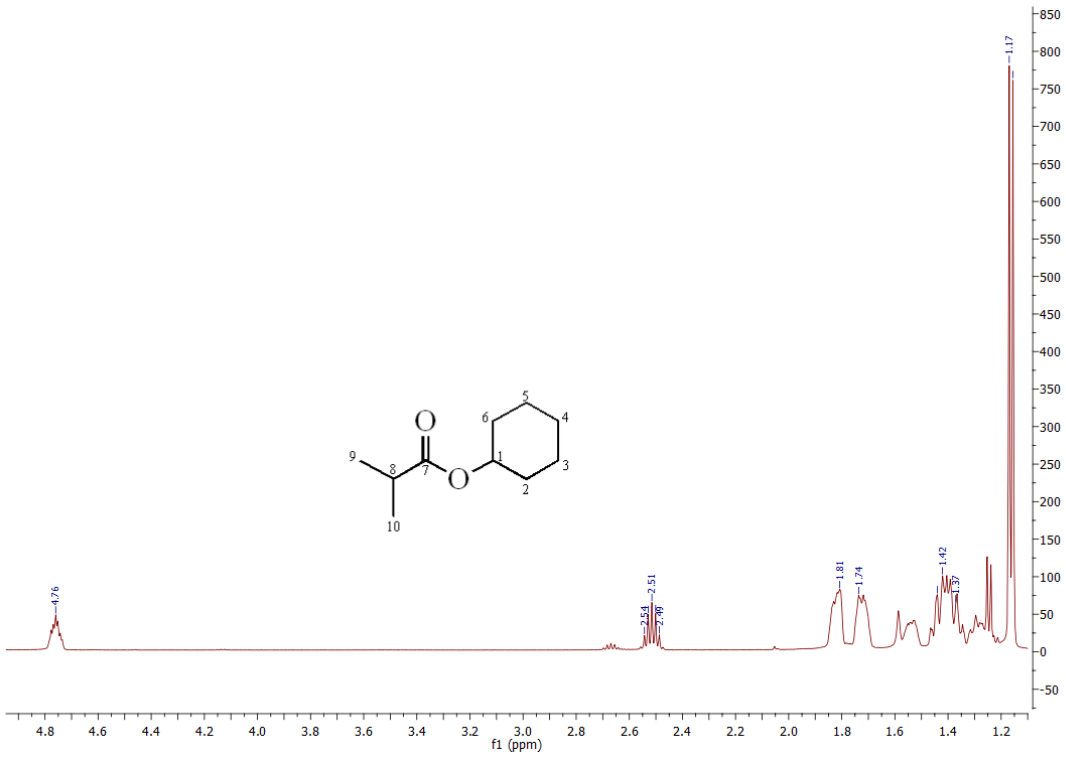


Figure S6 ^{1}H NMR of cyclohexyl isobutyrate.

NMR data for cyclooctyl Acetate

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.95-4.85 (m, 1H, H1), 1.99 (s, 3H, 3xH10), 1.83-1.42 (m, 14H).

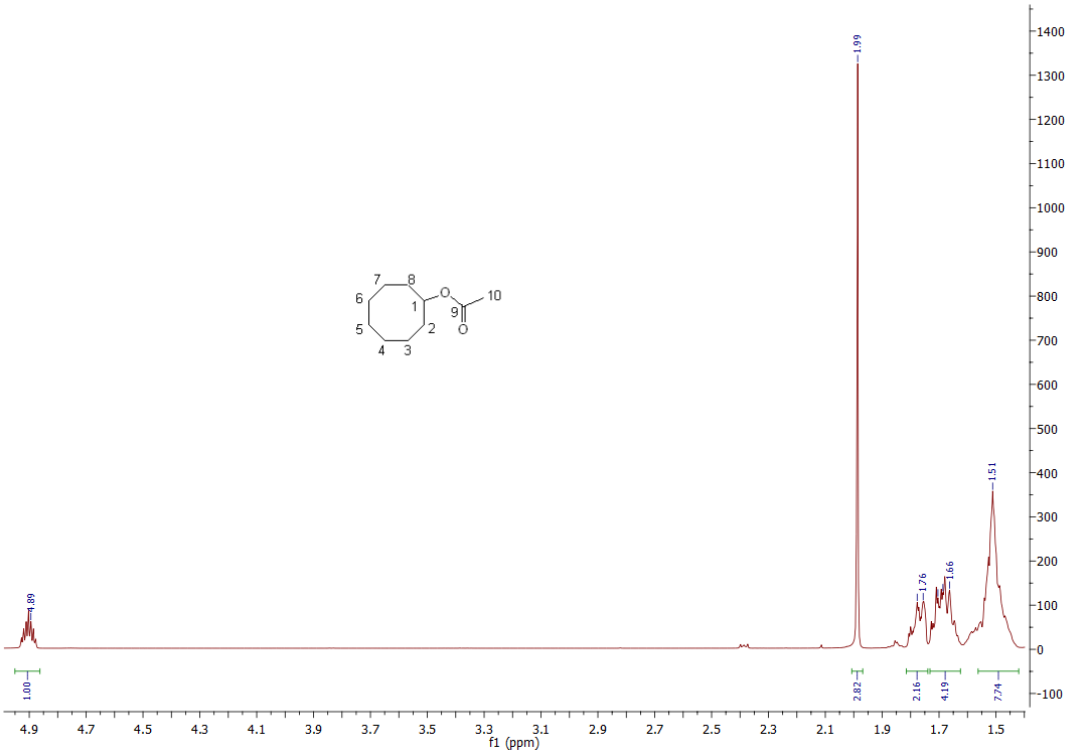


Figure S6 ^{1}H NMR of cyclooctyl acetate

NMR data for cyclooctyl isobutyrate

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.99-4.84 (m, 1H, H1), 2.49 (*septet*, J = 7.0 Hz, 1H, H10), 1.85-1.43 (m, 14H), 1.16 (d, J = 7.0 Hz, 6H, 3xH11 & 3xH12).

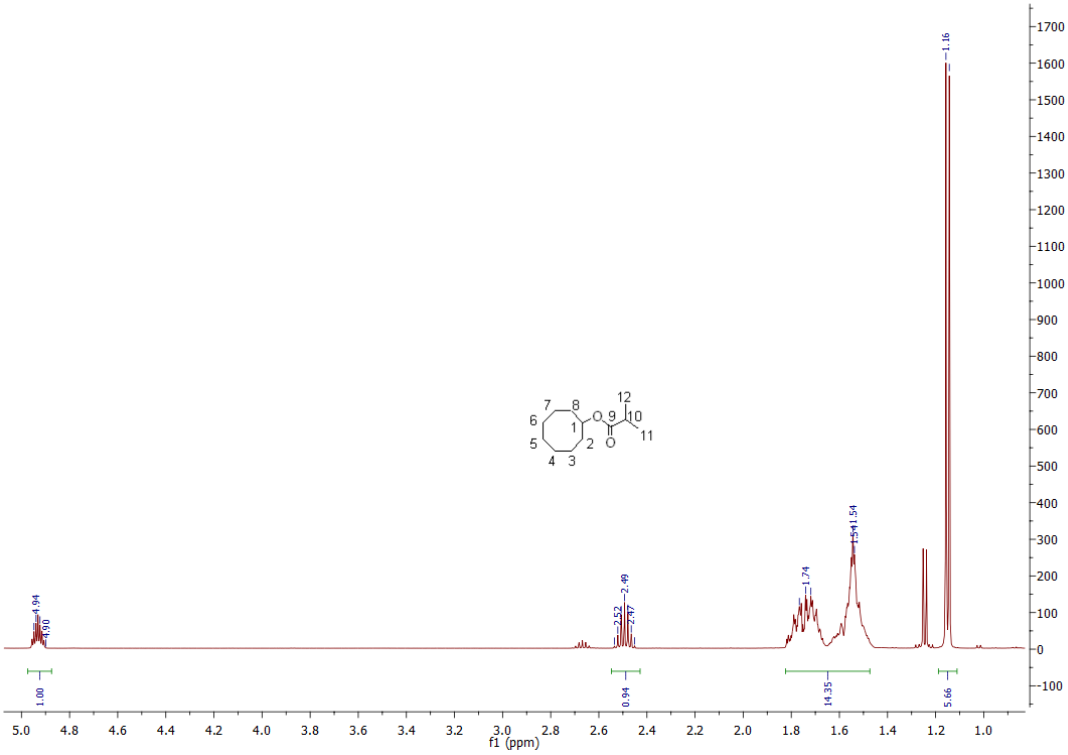


Figure S6 ^{1}H NMR of cyclooctyl isobutyrate.

NMR data for cyclododecyl acetate

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.05-4.95 (m, 1H, H1), 2.03 (s, 3H, 3xH14), 1.76-1.63 and 1.53-1.26 (m, 22H).

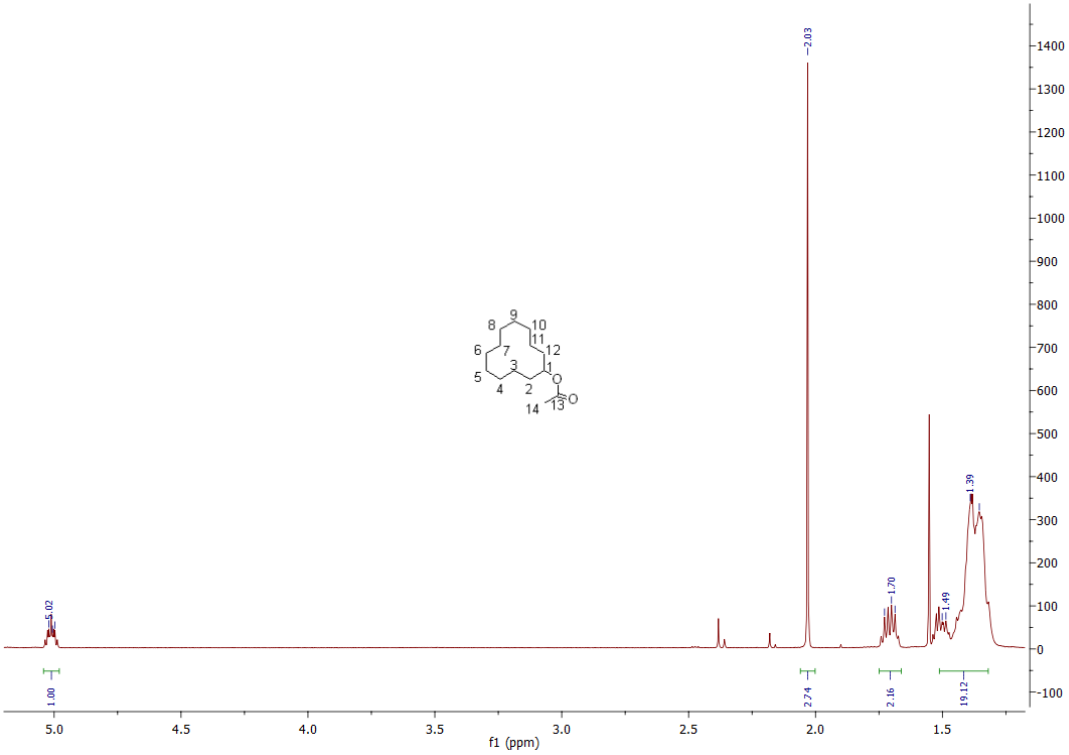


Figure S6 ^{1}H NMR of cyclododecyl acetate.

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