Productivity enhancement of C=C bioreduction by coupling the *in situ* substrate feeding product removal technology with isolated enzymes

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ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

Contents

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General methods

Materials. All chemical reagents and solvents were purchased from Sigma-Aldrich and used without further purification. With the only exception of horse liver alcohol dehydrogenase (HLADH, purchased from Sigma-Aldrich), all the enzymes employed were overexpressed in *Escherichia coli* BL21 (DE3) strains harboring a specific plasmid prepared according to standard molecular biology techniques: pET30a-OYE2 and pET30a-OYE3 from *Saccharomyces cerevisiae* BY4741 and pKTS-GDH from *Bacillus megaterium* DSM509.[1]

Analytical methods. GC-MS analyses were performed on an Agilent HP 6890 gas-cromatograph equipped with a 5973 mass detector and an Agilent HP-5 (30 m × 0.25 mm × 0.25 μm) column. Method: 60°C (1 min) / 6°C/min / 150°C (1 min) / 12°C/min / 280°C (5 min). Chiral GC analyses of compounds **1b**, **3b**, **4b** were performed on a DANI HT 86.10 gas-chromatograph equipped with a Varian Chirasil-Dex CB (25 m × 0.25 mm) column. Method for compound **1b**: 75°C (1 min) / 3°C/min / 119°C (17 min) / 30°C/min / 180°C (5 min). Method for compounds **3b** and **4b**: 60°C (1 min) / 2°C/min / 150°C (10 min) / 30°C/min / 180°C (5 min). Chiral GC analyses of compound **2b** were performed on an Agilent HP 6890 gas-chromatograph equipped with a Mega DAcTBSil.BetaCDX (25 m × 0.25 mm × 0.25 μm) column. Method: 60°C (3 min) / 3°C/min / 180°C (2 min) / 30°C/min / 220°C (5 min). Chiral HPLC analyses were performed on a Merck-Hitachi L-4250 chromatograph equipped with a Chiralcel OD column and UV detector (210 nm). For compound **1c**: mobile phase *n*-hexane/*i*-PrOH 98:2, flow rate 0.6 mL/min. For the methyl ester prepared from **1b**: mobile phase *n*-hexane/*i*-PrOH 99:1, flow rate 0.6 mL/min. For compound **2c**: mobile phase *n*-hexane/*i*-PrOH 98:2, flow rate 0.6 mL/min. For compounds **3c** and **4c**: mobile phase *n*-hexane/*i*-PrOH 97:3, flow rate 1.0 mL/min. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX 400 spectrometer (400 MHz ^{1}H, 100.6 MHz ^{13}C) in $CDCl_3$ solution at r.t., using TMS as internal standard for ^{1}H and $CDCl_3$ for ^{13}C; chemical shifts δ are expressed in ppm relative to TMS, *J* values are given in Hz. Optical rotations were determined on a Dr. Kernchen Propol digital automatic polarimeter and are expressed in ° cm^3 g^{-1} dm^{-1}. TLC analyses were performed on Merck Kieselgel 60 F_{254} plates. Protein concentration was determined with the Bio-Rad Protein Assay reagent according to Bradford,[2] using bovine serum albumine (BSA) as a standard.

[1] M. Bechtold, E. Brenna, C. Femmer, F. G. Gatti, S. Panke, F. Parmeggiani, A. Sacchetti, *Org. Process Res. Dev.*, 2011, DOI: 10.1021/op200085k.
[2] M.M. Bradford, *Anal. Biochem.*, 1976, **72**, 248.

Experimental procedures for the biocatalysed reductions

Baker's yeast-mediated bioreduction

Method A (homogenous phase). To a mechanically stirred mixture of commercial baker's yeast (250 g) in tap water (1 L) at 30°C, was added a solution of glucose (50 g) in water (100 mL). After 1 hour the substrate **1a** (1 g) was added in one portion. The vigorous stirring was continued for 4 days. During that time more baker's yeast (100 g) and glucose (20 g) were added after 24 and 48 hours. Then, the mixture was filtered on a celite pad and the aqueous phase was extracted with EtOAc (4 × 250 mL). The combined organic phase was concentrated under reduced pressure to afford a brownish oil that was dissolved in CH_2Cl_2 (150 mL). To this solution, after washing with brine (2 × 100 mL) and drying over Na_2SO_4, activated MnO_2 (20 g) was added. After complete conversion of the residual allylic alcohol to the corresponding aldehyde (checked by TLC), the MnO_2 was removed by filtration and the solution was concentrated under reduced pressure. The residue was submitted to column chromatography purification using *n*-hexane/EtOAc (9:1) as eluent to give, in order of elution, the starting material and the corresponding saturated alcohol.

Method B (SFPR technology). The same procedure of Method A is followed. The substrate **1a-4a** adsorbed on XAD 1180 resin (for substrate loading and $X_{r/s}$ see Table 1) was added in one portion. After 48 h, the mixture was filtered on a sintered glass funnel (porosity 0, >165 μm) and the aqueous phase was extracted again with more resin (10 g). The combined resin crops were washed with acetone (100 mL) and EtOAc (4 × 100 mL). The work-up was carried out as described above.

Enoate reductases-mediated bioreduction

Method A (OYEs). The substrate **1a-4a** either dissolved in DMSO or adsorbed on XAD 1180 resin (for substrate loading and $X_{r/s}$ see Table 1) was added to a solution of glucose (4 eq. with respect to **1a-4a**), $NADP^+$ (0.1 mM), GDH (4 U mL^{-1}) and OYE (150 μg mL^{-1}) in phosphate buffer (1.0÷10.0 mL, 50 mM, pH 7.0). The mixture was stirred for 12 h in an orbital shaker (160 rpm, 30°C). The solution was decanted and both the resins and the aqueous phase were extracted with EtOAc (2 × 0.5 mL/mL_{aq}), centrifuging after extraction (15000 *g*, 1.5 min). The combined organic solutions were dried over Na_2SO_4 and concentrated under reduced pressure, yielding the saturated aldehyde or a mixture of saturated aldehyde and starting material. Reactions in optimized conditions were scaled up to preparative scale (50÷150 mg) for product characterization and determination of isolated yields.

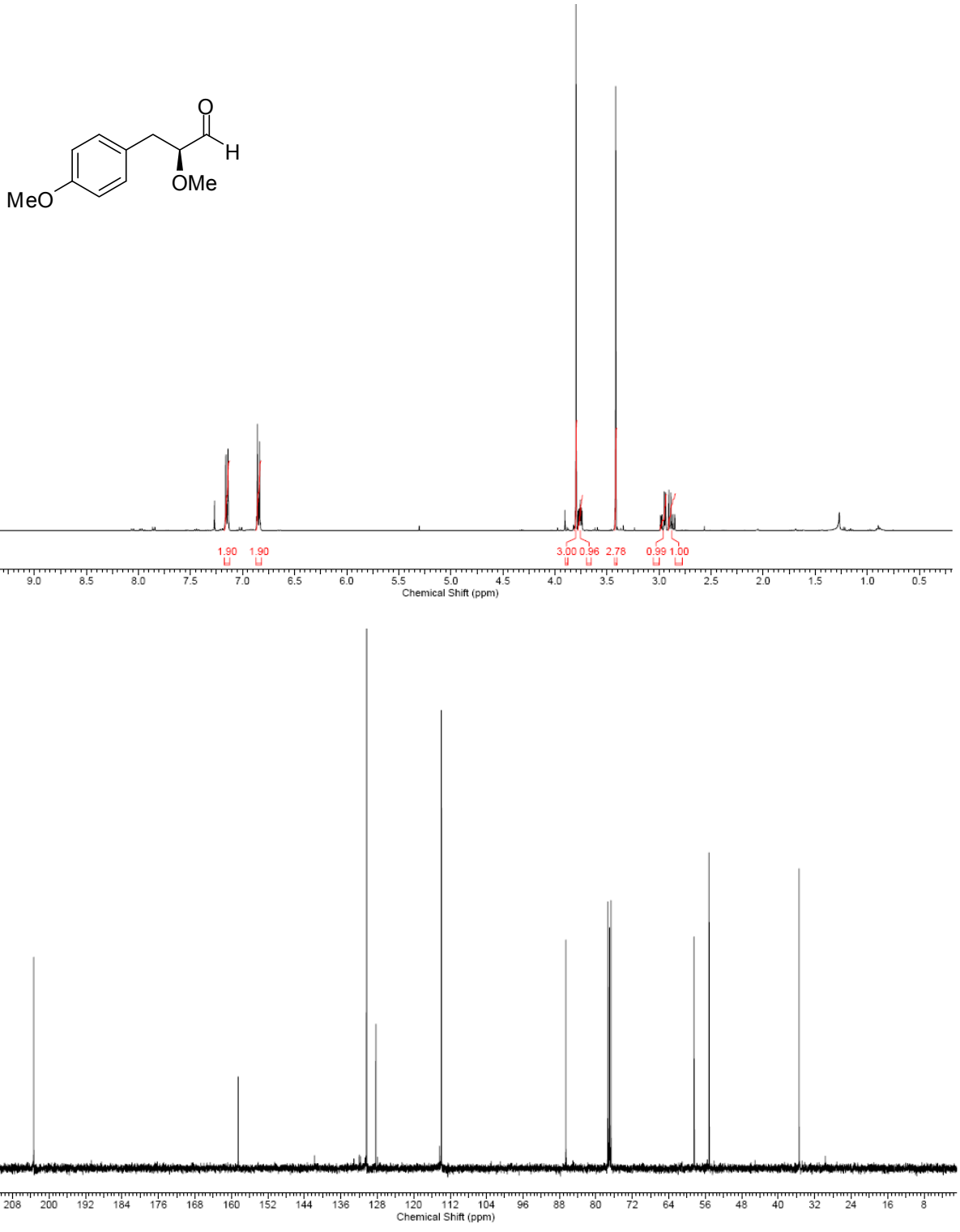
Method B (OYEs+HLADH). The same procedure of Method A is followed, adding HLADH (2 U mL^{-1}) and NAD^+ (0.1 mM) to the reaction mixture.

Experimental procedure for the overexpression of the enzymes in *E. coli* BL21 (DE3)

A 5 mL culture in LB medium containing the appropriate antibiotic (50 μg mL^{-1} kanamycin for pET-30a, 100 μg mL^{-1} ampicillin for pKTS) was inoculated with a single colony from a fresh plate and grown overnight at 37°C and 220 rpm. This starter culture was used to inoculate a 200 mL culture, which was in turn grown overnight at the same conditions and used to inoculate a 1.5 L culture. The latter was shaken at 37°C and 220 rpm until OD_{600} reached 0.4-0.5 and then enzyme expression was induced by adding 0.1 mM IPTG (50 ng mL^{-1} anhydrotetracycline was also added in the case of the pKTS-GDH plasmid). After 5-6 h the cells were harvested by centrifugation (5000 *g*, 20 min, 4°C), resuspended in 50 mL of lysis buffer (20 mM phosphate buffer pH 7.0, 300 mM NaCl, 10 mM imidazole) and homogenized (Haskel high-pressure homogenizer). The cell-free extract, after centrifugation (20000 *g*, 20 min, 4°C), was chromatographed on IMAC stationary phase (Ni-Sepharose Fast Flow, GE Healthcare) with a mobile phase composed of 20 mm phosphate buffer, pH 7.0, 300 mM NaCl and a 10-300 mM imidazole gradient. Protein elution was monitored at 280 nm, the fractions were collected according to the chromatogram and dialyzed twice against 1.0 L of 20 mM phosphate buffer pH 7.0 (12 h each, 4°C) to remove imidazole and salts. Purified protein aliquots were stored frozen at –80°C.

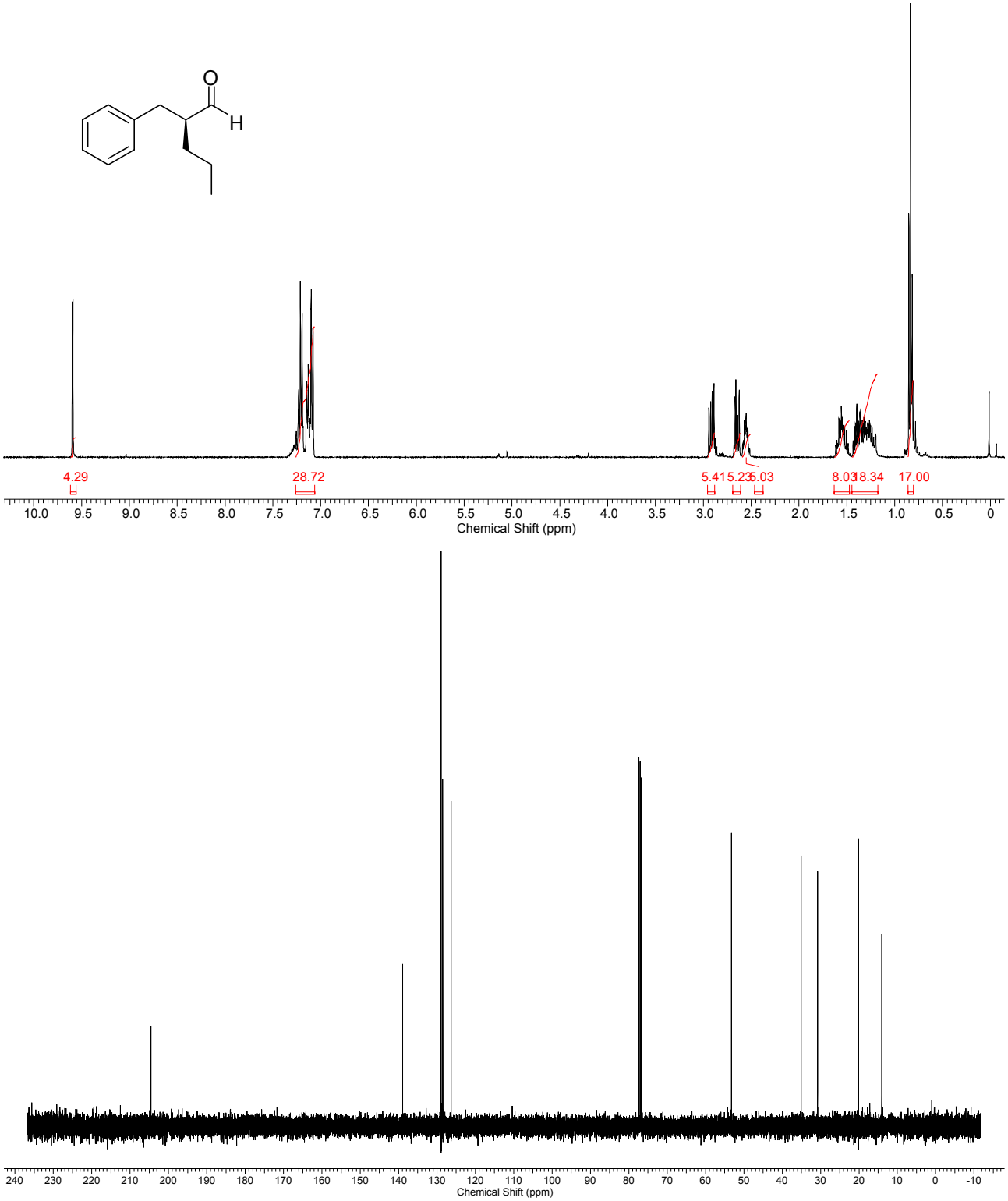
(*S*)-2-methoxy-3-(4-methoxyphenyl)propanal (1b)

$[\alpha]_D^{20}$ = – 21.6 (*c* 1.14, $CHCl_3$), *ee* 94%; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.68 (d, *J* = 1.8 Hz, 1 H), 7.15 (d, *J* = 8.5 Hz, 2 H), 6.85 (d, *J* = 8.5 Hz, 2 H), 3.80 (s, 3 H), 3.76 (ddd, *J* = 7.6, 5.2, 2.2 Hz, 1 H), 3.42 (s, 3 H), 2.79-3.01 (m_{AB}, 2 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 203.3, 158.5, 130.3, 128.3, 113.9, 86.6, 58.5, 55.2, 35.5. HRMS (ESI) calcd for $C_{11}H_{14}O_3$ 194.0943, found 194.0941.



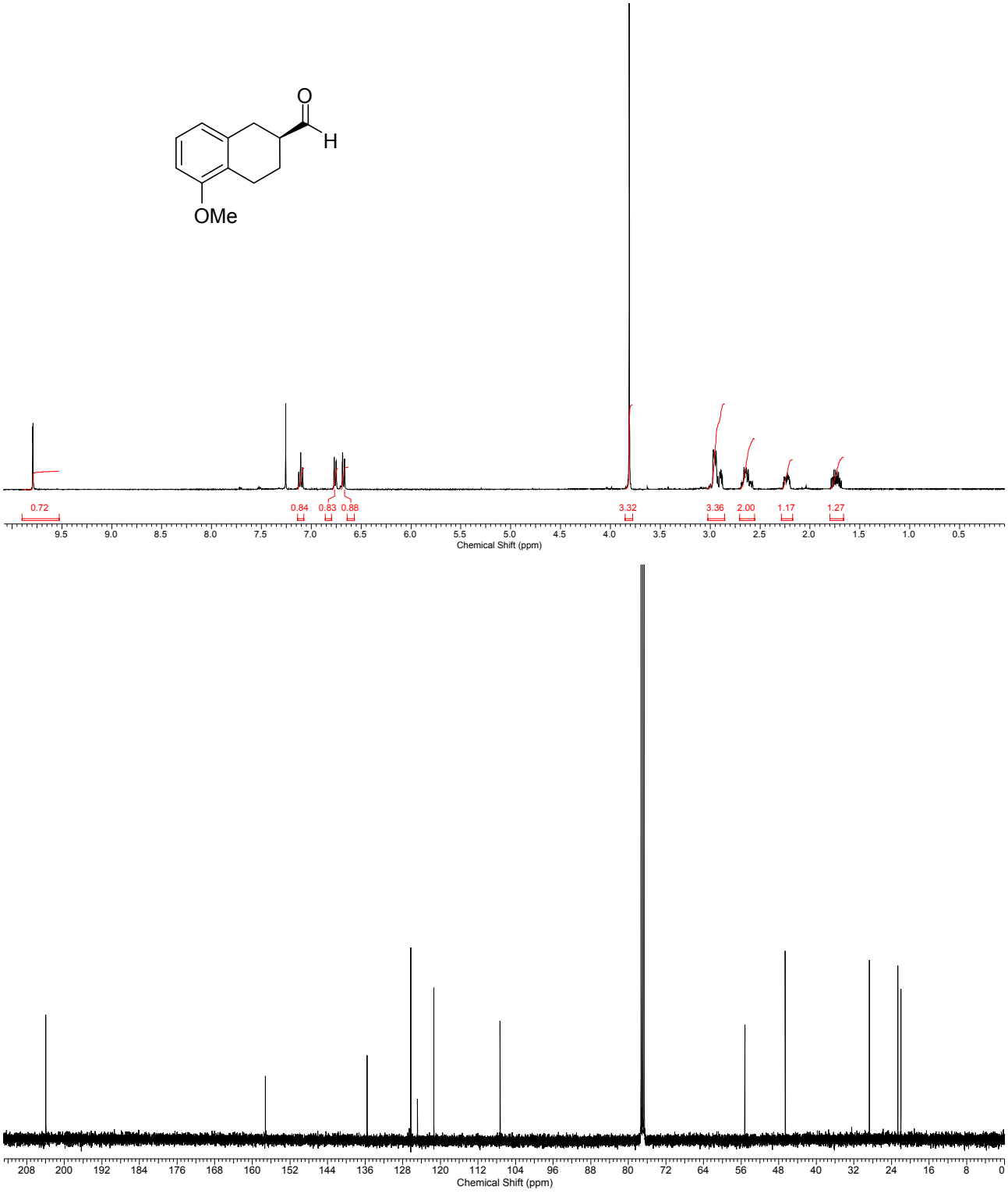
(*S*)-2-benzylpentanal (2b)

$[\alpha]_D^{20}$ = – 7.0 (*c* 1.1, $CHCl_3$), *ee* 90%; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.59 (d, J = 2.5 Hz, 1 H), 7.06-7.27 (m, 5 H), 2.78 (m_{AB}, 2 H), 2.51-2.60 (m, 1 H), 1.17-1.63 (m, 4 H), 0.83 (t, J = 7.6 Hz, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 204.6, 138.9, 128.9, 128.5, 126.3, 53.2, 35.1, 30.8, 20.2, 14.0. HRMS (ESI) calcd for $C_{12}H_{16}O$ 176.1201, found 176.1199.



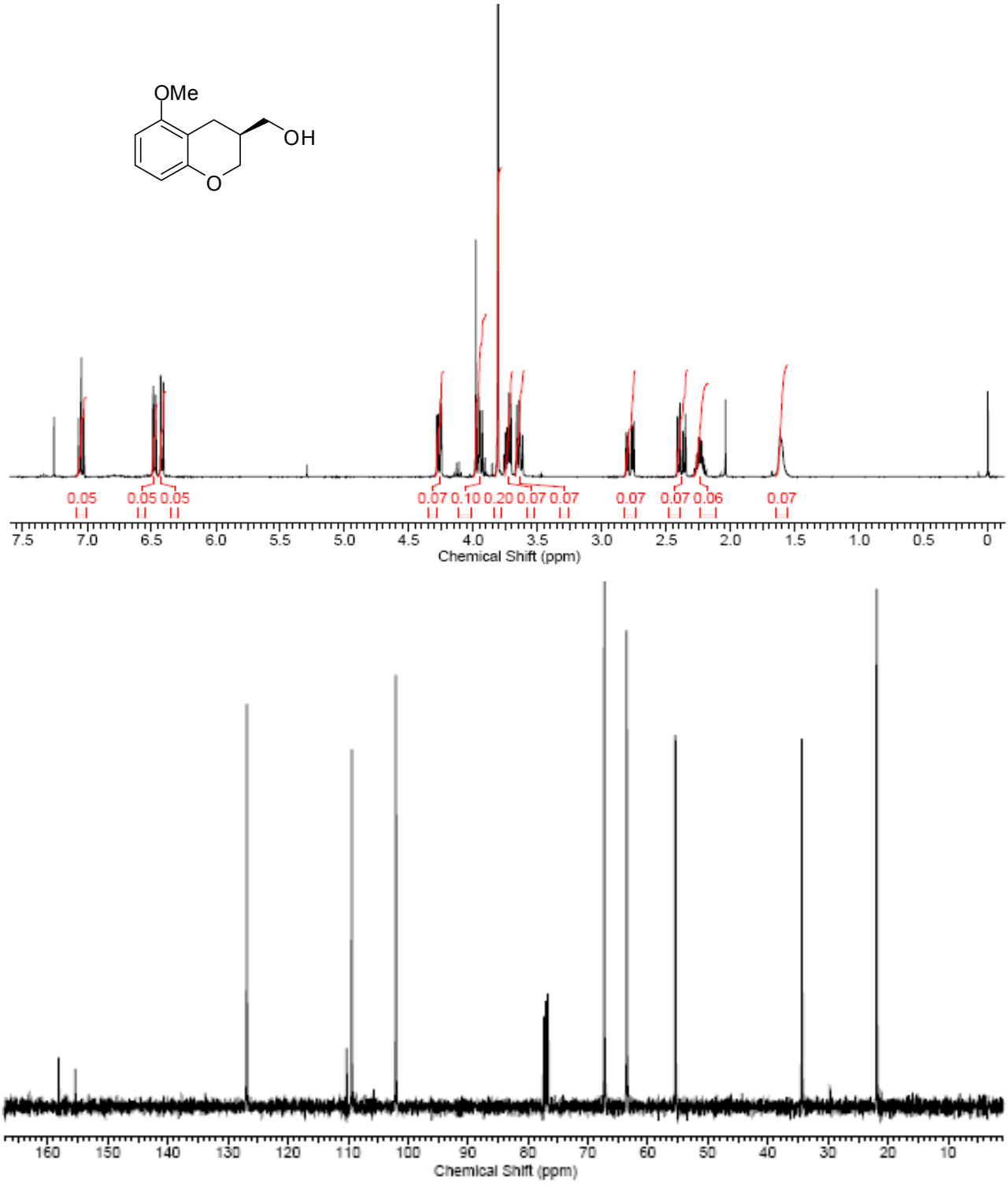
(*S*)-5-methoxy-1,2,3,4-tetrahydronaphthalene-2-carbaldehyde (3b)

$[\alpha]_D^{20}$ = – 5.0 (*c* 1.0, $CHCl_3$), *ee* 73%; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.79 (d, *J* = 1.2 Hz, 1 H), 7.11 (t, *J* = 8.0 Hz, 1 H), 6.76 (d, *J* = 7.4 Hz, 1 H), 6.68 (d, *J* = 7.8 Hz, 1 H), 3.81 (s, 3 H), 2.87-3.01 (m, 3 H), 2.56-2.70 (m, 2 H), 2.19-2.28 (m, 1 H), 1.68-1.80 (m, 1 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 203.9, 157.2, 135.6, 126.3, 124.9, 121.4, 107.3, 55.2, 46.6, 28.7, 22.7, 22.0. HRMS (ESI) calcd for $C_{12}H_{14}O_2$ 190.0994, found 190.0991.



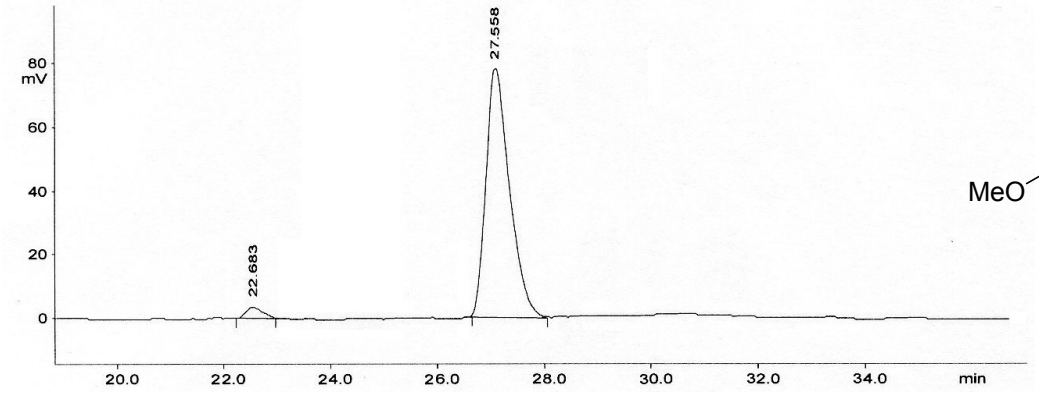
(*S*)-(5-methoxychroman-3-yl)methanol (4c)

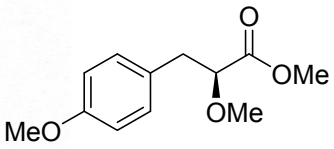
$[\alpha]_D^{20}$ = – 6.2 (*c* 1.17, $CHCl_3$), *ee* 98%; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.05 (t, *J* = 8.2, Hz, 1 H), 6.48 (d, *J* = 8.2 Hz, 1 H), 6.42 (d, *J* = 8.2 Hz, 1 H), 4.27 (ddd, *J* = 10.7, 3.1, 1.4 Hz, 1 H), 3.95 (dd, *J* = 10.7, 7.7 Hz, 1 H), 3.81 (s, 3 H), 3.73 (dd, *J* = 10.8, 5.8 Hz, 1 H), 3.64 (dd, *J* = 10.8, 7.8 Hz, 1 H), 2.79 (dd, *J* = 17.1, 6.0 Hz, 1 H), 2.38 (dd, *J* = 17.1, 7.8 Hz, 1 H), 2.29-2.19 (m, 1 H), 1.62 (br s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$ δ ppm 158.2, 155.4, 126.9, 110.2, 109.4, 102.0, 67.3, 63.6, 55.4, 34.4, 21.9. HRMS (ESI) calcd for $C_{11}H_{14}O_3$ 194.0943, found 194.0948.



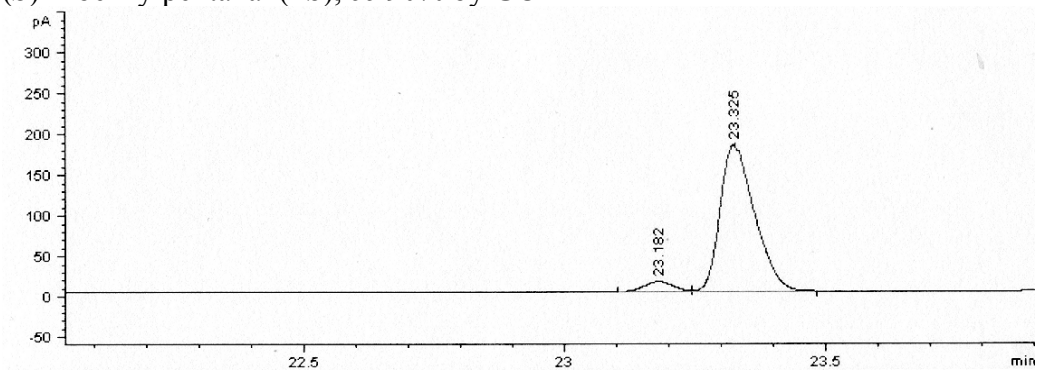
Representative GC/HPLC chromatograms

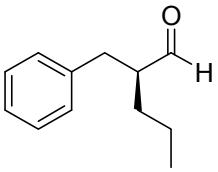
Methyl (*S*)-2-methoxy-3-(4-methoxyphenyl)propanoate (prepared from **1b**), *ee* 94% by HPLC



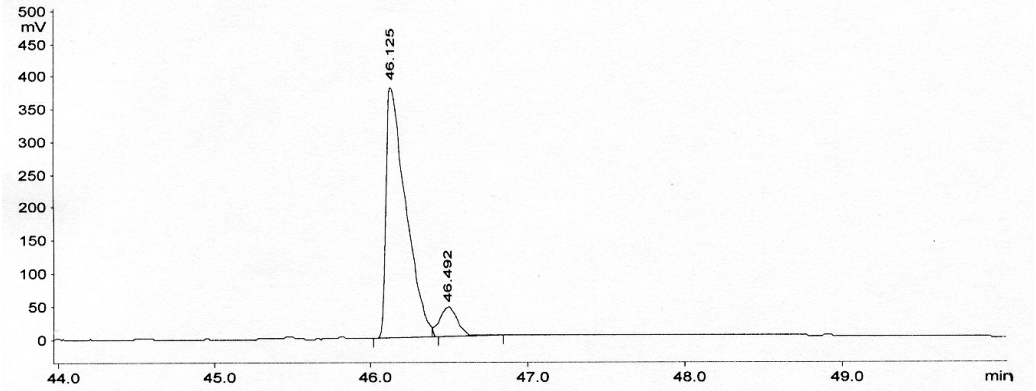


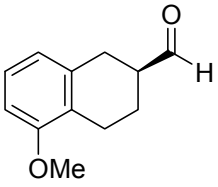
(*S*)-2-benzylpentanal (**2b**), *ee* 90% by GC





(*S*)-5-methoxy-1,2,3,4,-tetrahydronaphthalene-2-carbaldehyde (**3b**), *ee* 83% by GC





(*S*)-(5-methoxychroman-3-yl)methanol (**4c**), *ee* 98% by HPLC