Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

(*S*)-Selective MenD variants from *Escherichia coli* provide access to new functionalized chiral α-hydroxy ketones

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1. General experimental procedures

All chemical reagents, solvents, buffers, salts, α-ketoglutaric acid and benzaldehyde derivatives were purchased from Sigma-Aldrich and used without further purification. KOD Hot Start DNA Polymerase was obtained from Merck Millipore and the restriction enzyme *DpnI* from (Thermo Scientific).

Bradford assays were measured at 595 nm on a UV mini – 1240 spectrophotometer (SHIMADZU). The **optical density** (OD) of bacterial cultures was determined at 600 nm on a UV – 1601 spectrophotometer (SHIMADZU). **GC-MS analyses** were performed on a HP 6890 N Series GC system (EI, 70 eV) with a HP 5973 Network Mass Selective Detector (Agilent), equipped with a DB-5MS column (Agilent, 30 m × 0.25 mm, 0.25 µm thick stationary phase), using the following conditions: injector temp: 250 °C, detector temp: 300 °C, flow rate: 25 mL min^{-1}, temp program: 60 ºC for 3 min, then 20 ºC min^{-1} to 280 ºC for 4 min. **Nuclear magnetic resonance** (NMR) spectra were measured on a DRX 400 instrument (Bruker) operating at 400 and 100 MHz for ^{1}H and ^{13}C acquisitions, respectively. Chemical shifts (δ) of ^{1}H and ^{13}C NMR spectra are reported in ppm with a solvent resonance as an internal standard (^{1}H NMR: $CHCl_3$ 7.24 ppm, CHD_2OD 3.30 ppm; ^{13}C NMR: $CDCl_3$ 77.0 ppm, CD_3OD 49.0 ppm). Coupling constants (*J*) are reported in hertz (Hz). Splitting patterns are indicated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet. **High-performance liquid chromatography** (HPLC-DAD) was performed on a HP 1100 and on a 1260 Infinity chromatography system (Agilent), respectively. Enantiomeric excesses were determined using the columns Diacel Chiralcel OB 10 µm (250 mm × 4.6 mm), Diacel Chiralcel OD-H 5 µm (250 mm × 4.6 mm) and Chiralpac IC 5 µm (250 mm × 4.6 mm). **Circular dichroism** (CD) spectra were recorded in order to determine absolute configuration of the products using a Jasco J-810 spectrometer (Jasco International Co).

2. Structural investigation of *Ec*MenD and docking experiments

Thiamine diphosphate (ThDP)-dependent enzymes are well known for their catalytic potential to form various chiral α-hydroxy ketones. Most of the wild type (wt) enzymes are (*R*)-selective in the carboligation reaction, whereby the access to (*S*)-α-hydroxy ketones is limited. Mainly steric properties of the active site of ThDP-dependent enzymes are responsible for the stereoselectivity. Prerequisite for (*S*)-selectivity is a structural element called an *S*-pocket, which allows an antiparallel arrangement of donor and acceptor substrate prior to carboligation.[1] *S*-pockets are present in most ThDP-dependent enzymes, however, not accessible for acceptor substrates due to large amino acid side chains.[2,3]

The carboligation potential of MenD from *Escherichia coli* (*Ec*MenD) was recently investigated in detail concerning substrate diversity and stereoselectivity. Carboligation of α-ketoglutaric acid (**1**) as donor with different aromatic aldehydes results in the formation of α-hydroxy ketones with high enantiomeric excesses (*ee*) of >93% (*R*).[4] To explain the high (*R*)-selectivity of *Ec*MenD, the structure (pdb: 2JLC)[5] was investigated concerning important amino acid residues in the *S*-pocket region in the first step using the PyMOL software (Schrödinger). Five residues were identified in the *S*-pocket region: P30, G31, S32, I474, and F475 (Fig. S1 A). Docking of benzaldehyde (**2a**) revealed that obviously only a parallel arrangement of **2a** and **1** is possible meaning that both side chains are oriented towards the substrate channel. An antiparallel arrangement of **2a** would lead to a clash with the amino acid side chains of the *S*-pocket region (Fig. S1 B) explaining the high (*R*)-selectivity in the reaction of **1** and **2a** (*ee* = 99%).

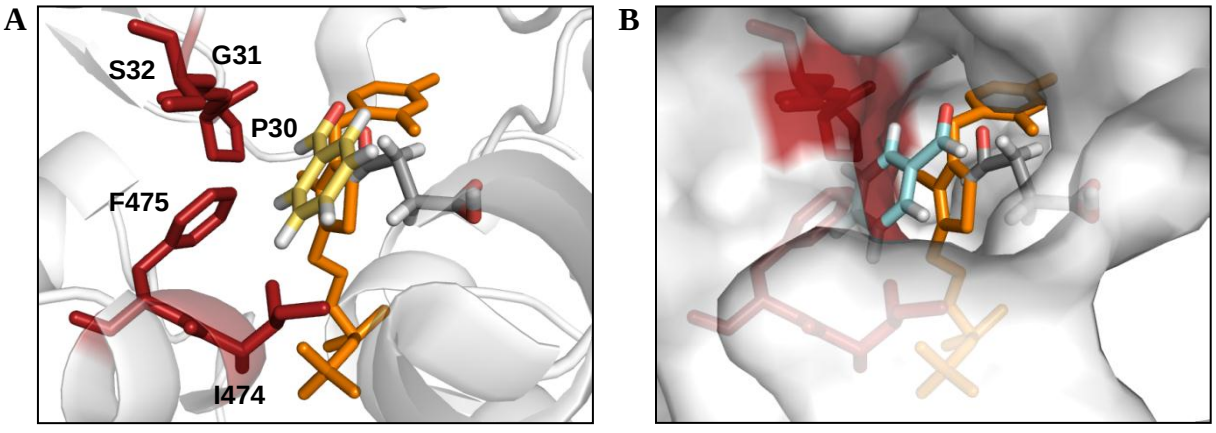


Fig. S1: Amino acids (red) defining the *S*-pocket of *Ec*MenD (**A**) and the resulting structure of the acceptor-binding site (**B**). Only a parallel arrangement of the acceptor benzaldehyde (yellow) to the donor α-ketoglutaric acid (gray) is possible. An antiparallel arrangement of benzaldehyde (cyan) would lead to a clash with the amino acids of the *S*-pocket region. The cofactor ThDP is colored in orange.

Among the five amino acids of the *S*-pocket region, I474 and F475 were deduced to be crucial for stereoselectivity. Both amino acids prevent the antiparallel arrangement of **2a** prior to C-C-bond formation (Fig. S2 A). However, a replacement of both with the smaller amino acids glycine and alanine, respectively, opens the *S*-pocket for the phenyl ring of **2a** (Fig. S2 B, exemplarily shown for variant I474A/F475G) allowing the antiparallel arrangement of donor and acceptor substrate. The new resulting *S*-pocket should be able to stabilize **2a**, thus increasing the formation of the respective (*S*)-product. The experimental data (see Communication) support this model. Stereoselectivity of *Ec*MenD could be inverted by mutation of I474 and F475 to glycine and alanine, respectively, yielding the (*S*)-selective variants I474G/F475G, I474A/F475G, I474G/F475A, and I474A/F475A with *ee*s of 27 – 75% (*S*) in the reaction of **1** and **2a**.

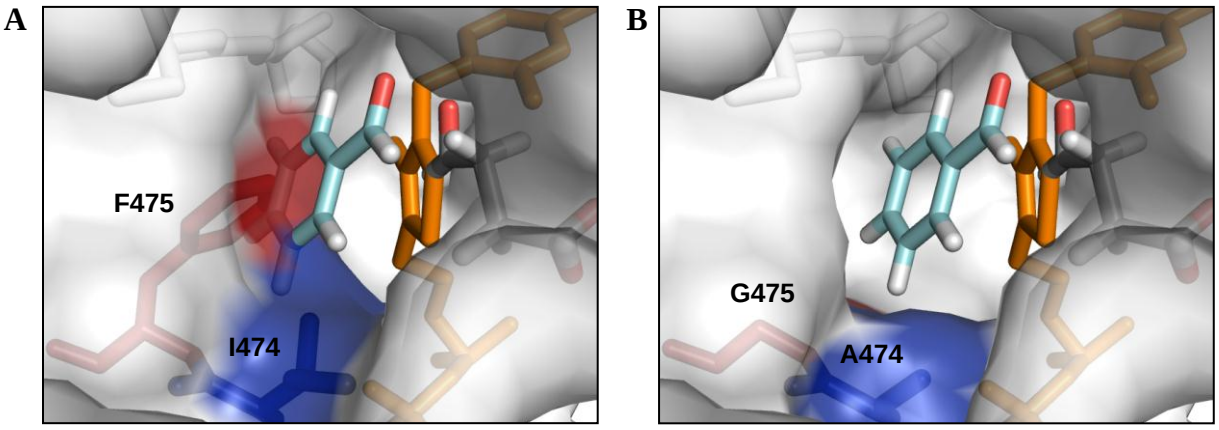


Fig. S2: The antiparallel orientation of benzaldehyde modeled inside the active side of *Ec*MenDwt (**A**) and *Ec*MenDI474A/F475G (**B**). The exchange of I474 (blue) to alanine and F475 (red) to glycine allows the antiparallel arrangement of benzaldehyde (cyan) to the donor α-ketoglutaric acid (gray). The cofactor ThDP is colored in orange.

3. Construction, expression and purification of *Ec*MenD variants

The original *menD* gene of *Escherichia coli* K12 was cloned into pET-19b (Novagen) providing an N-terminal His_{10}-tag.[4] Site-directed mutagenesis was performed using the standard QuikChange® protocol from Stratagene. However, the KOD Hot Start DNA Polymerase (Merck Millipore) was used in the PCR reaction. First, *Ec*MenDF475G was prepared using the forward (fw) 5'-CAACAACGGCGGGCAAATTggCTCGCTGTTGC-3' and reverse (rv) primer 5'-GCAACAGCGAGccAATTTGCCCGCCGTTGTTG-3'. The mutated codons are underlined, with lower-case letters indicating base changes relative to the template. Based on *Ec*MenDF475G the double mutants *Ec*MenDI474G/F475G and *Ec*MenDI474A/F475G were prepared. *Ec*MenDI474A/F475G served as a template for the construction of *Ec*MenDI474A/F475A, whereas *Ec*MenDI474G/F475A was prepared based on *Ec*MenDI474G/F475G. The following fw and rv primers were used for mutagenesis:

*Ec*MenDI474G/F475G: 5'-CAACAACGGCGGGCAAggcGGCTCGCTGTTGC-3' (fw)
5'-GCAACAGCGAGCCcggTTGCCCGCCGTTGTTG-3' (rv)

*Ec*MenDI474A/F475G: 5'-CAACAACGGCGGGCAAgcgGGCTCGCTGTTGCC-3' (fw)
5'-GGCAACAGCGAGCCcgcTTGCCCGCCGTTGTTG-3' (rv)

*Ec*MenDI474G/F475A: 5'-CAACGGCGGGCAAGGCgcCTCGCTGTTGCCAAC-3' (fw)
5'-GTTGGCAACAGCGAGgcGCCTTGCCCGCCGTTG-3' (rv)

*Ec*MenDI474A/F475G: 5'-CAACGGCGGGCAAGCGGcgTCGCTGTTGCCAAC-3' (fw)
5'-GTTGGCAACAGCGAcgCCGCTTGCCCGCCGTTG-3' (rv).

Following mutagenesis, the template DNA was digested by *DpnI*. Finally, *E. coli* BL21(DE3) was transformed with the remaining PCR products. Gene sequences were confirmed by DNA sequencing (LGC Genomics).

Expression and purification of *Ec*MenD and its variants was carried out with minor modifications as described elsewhere.[4] After 14 h of cultivation at 120 rpm and 20 °C cells were pelleted by centrifugation at 8000 rpm and 4 °C for 30 min using Avanti J-20 XP, rotor JLA-8.1000 (Beckman Coulter). The cell pellet was resuspended in lysis buffer (20% w/v) containing lysozyme (1 mg/mL). After cell disruption by sonication cell debris was harvested by centrifugation at 20.000 rpm and 4 °C for 45 min using rotor JA-20 (Beckman Coulter). Purification of His-tagged all *Ec*MenD variants was performed by immobilized nickel chelate chromatography. The resulting enzyme was first desalted by size exclusion chromatography using Sephadex™ G25M (GE Healthcare) and finally freeze-dried using ALPHA 2-4

(Christ). Lyophilized *Ec*MenD variants were stored at -20 °C for several months without significant loss of activity.

4. Reaction conditions for carboligation reactions with *Ec*MenD variants

Lyophilized *Ec*MenD variants were used for all preparations. The protein concentration was determined according to Bradford.[6] Reactions were performed in phosphate buffer (50 mM potassium phosphate, 0.1 mM ThDP, 2 mM $MgCl_2 \cdot 6H_2O$, pH 8.0) at 30 ºC and 300 rpm using a Thermomixer (Eppendorf). The final concentration of MenD was set to 700 µg/mL. 50 mM α-ketoglutaric acid and 20 mM benzaldehyde derivatives were incubated with enzyme in 1.5 mL reaction buffer containing 5% (v/v) MTBE for 24 h. Negative control experiments without enzyme were performed to verify enzyme-catalyzed C-C bond formation.

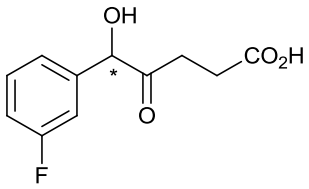
5. Analytical procedures

For GC-MS analysis 100 µL of the reaction mixture was acidified with 5 µL of 6 M HCl and extracted with 200 µL of ethyl acetate. For NMR analysis the complete reaction mixture of two 1.5 mL approaches was first extracted with 50 µL of 6 M HCl and then three times with 300 µL methyl *tert*-butyl ether (MTBE). Afterwards, MTBE was evaporated and the residual mixture was dissolved in 100 µL n-hexane/2-propanol (50:50). The products were purified by semi-preparative HPLC on a Reprosil Chiral-OM 5 µm (250 mm × 10 mm) column using the following conditions: n-hexane/2-propanol = 90:10 (0.25% trifluoroacetic acid (TFA)), 0.75 mL min^{-1} and 20 °C. Finally, the organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. NMR analyses were performed in $CDCl_3$. Determination of the enantiomeric excess was carried out on chiral phase HPLC. 200 µL of the reaction mixture was extracted with 10 µL 6 M HCl and 200 µL of MTBE. CD spectroscopy was performed in order to confirm the absolute configuration of the product. (*R*)-Phenylacetylcarbinol was used as reference compound. The reaction mixture was directly diluted 1:10 in ddH_2O and measured in a 2 mm cuvette. The spectra were recorded in the range of 240 - 350 nm. A blank buffer was measured to ensure that no background signal was present. (*R*)-PAC derivatives led to the appearance of a negative CD band with the maximum near 280 nm, whereas (*S*)-PAC derivatives led to the respective positive CD signal.

6. Characterization data for selected compounds

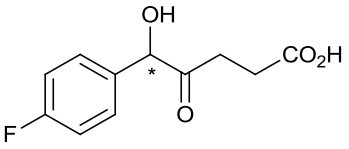
Complete characterizations of compounds **3a**, **b**, and **e - g** are given in Kurutsch *et al*. (2009) and Beigi *et al*. (2012). New compounds are described in the following.

5-(3-Fluorophenyl)-5-hydroxy-4-oxopentanoic acid **(3c)**:[4]



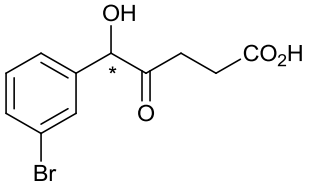
$C_{11}H_{11}FO_4$, M_r 226.20; ^{1}H NMR ($CDCl_3$): δ = 2.48–2.62 (m, 2H), 2.67–2.79 (m, 2H), 5.16 (s, 1H), 7.02–7.07 (m, 2H), 7.15 (d, 1H, J = 7.7 Hz), 7.34–7.37 (m, 2H) ppm; ^{13}C NMR ($CDCl_3$): δ = 27.5 (CH_2), 32.4 (CH_2), 79.1 (CH), 114.3 (d, CH, J = 22 Hz), 115.9 (d, CH, J = 21 Hz), 123.1 (d, CH, J = 3 Hz), 130.6 (d, CH, J = 8 Hz), 140.2 (C), 163.1 (d, CF, J = 248 Hz), 176.8 (COOH), 207.1 (C=O) ppm; HPLC-DAD: (Daicel Chiralpak IC 5 µm, 20 ºC, 1.2 mL min^{-1}: *n*-hexane/2-propanol (0.25% TFA) = 94.5:5.5): t_R(*R*) = 26.6 min, t_R(*S*) = 25.4 min.

5-(4-Fluorophenyl)-5-hydroxy-4-oxopentanoic acid **(3d)**:[4]



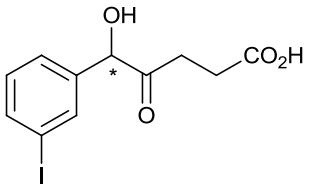
$C_{11}H_{11}FO_4$, M_r 226.20; ^{1}H NMR ($CDCl_3$): δ = 2.45–2.75 (m, 4H), 5.15 (s, 1H), AA'BB' spin system: 7.05-7.11 (m, BB', 2H, J_{HH} = $J_{HF(ortho)}$ =8.6 Hz), 7.29-7.34 (m, AA', 2H, J_{HH} = 8.6, $J_{HF(meta)}$ = 5.2 Hz) ppm; ^{13}C NMR ($CDCl_3$): δ = 27.5 (CH_2), 32.3 (CH_2), 79.0 (CH), 116.0 (d, CH, J = 22 Hz), 129.2 (d, CH, J = 8 Hz), 133.5 (d, C, J = 3 Hz), 163.0 (d, CF, J = 248 Hz), 176.9 (COOH), 207.5 (C=O) ppm; HPLC-DAD: (Daicel Chiralcel OD-H 5 µm, 20 ºC, 0.75 mL min^{-1}: *n*-hexane/2-propanol (0.25% TFA) = 95:5): t_R(*R*) = 41.6 min, t_R(*S*) = 44.5 min.

5-(3-Bromophenyl)-5-hydroxy-4-oxopentanoic acid **(3h)**:



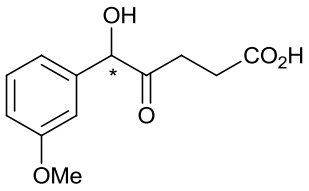
$C_{11}H_{11}BrO_4$, M_r 287.11; ^{1}H NMR ($CDCl_3$): δ = 2.47–2.63 (m, 2H), 2.67–2.78 (m, 2H), 5.12 (s, 1H), 7.24–7.30 (m, 2H), 7.46–7.52 (m, 2H) ppm; ^{13}C NMR ($CDCl_3$): δ = 27.5 (CH_2), 32.4 (CH_2), 79.1 (CH), 123.1 (CBr), 126.0 (CH), 130.4 (CH), 130.6 (CH), 132.0 (CH), 139.9 (C), 176.9 (COOH), 207.0 (C=O) ppm; GC-MS (EI): t_R = 10.2 min, *m/z* (%) 244 (6) $[M - CO_2]^+$, 242 (6) $[M - CO_2]^+$, 185 (31) $[C_7H_4BrO]^+$, 183 (100) $[C_7H_4BrO]^+$, 157 (31) $[C_6H_4Br]^+$, 155 (31) $[C_6H_4Br]^+$, 76 (20) $[C_6H_4]^+$, 57 (25) $[C_3H_5O]^+$; $t_{R(lactone)}$ = 13.2 min, *m/z* (%) 185 (100) $[C_7H_4BrO]^+$, 183 (100) $[C_7H_4BrO]^+$, 157 (25) $[C_6H_4Br]^+$, 155 (25) $[C_6H_4Br]^+$, 85 (63) $[C_4H_5O_2]^+$; HPLC-DAD: (Daicel Chiralpac IC 5 µm, 40 ºC, 1.2 mL min^{-1}: *n*-hexane/2-propanol (0.25% TFA) = 95.5:4.5): t_R(*R*) = 38.9 min, t_R(*S*) = 40.1 min.

5-Hydroxy-5-(3-iodophenyl)-4-oxopentanoic acid **(3i)**:[4]



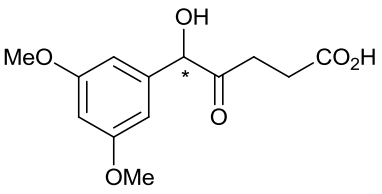
$C_{11}H_{11}IO_4$, M_r 334.11; HPLC-DAD: (Daicel Chiralpac IC 5 µm, 20 ºC, 1.2 mL min^{-1}: *n*-hexane/2-propanol (0.25% TFA) = 94.5:5.5): $t_R(R)$ = 28.1 min, $t_R(S)$ = 31.2 min.

5-Hydroxy-5-(3-methoxyphenyl)-4-oxopentanoic acid **(3j)**:[4]



$C_{12}H_{14}O_5$, M_r 238.24; ^{1}H NMR ($CDCl_3$): δ = 2.51–2.59 (m, 2H), 2.66–2.76 (m, 2H), 3.80(s, 3H) 5.13 (s, 1H), 6.84–6.93 (m, 3H), 7.30 (t, 1H, *J* = 7.9 Hz) ppm; ^{13}C NMR ($CDCl_3$): δ = 27.5 (CH_2), 32.4 (CH_2), 55.3 (CH_3), 79.6 (CH), 112.6 (CH), 114.6 (CH), 119.8 (CH), 130.3 (CH), 139.2 (C), 160.1 (C), 177.0 (COOH), 207.6 (C=O) ppm; HPLC-DAD: (Daicel Chiralcel OB 10 µm, 20 ºC, 1.0 mL min^{-1}: *n*-hexane/2-propanol (0.25% TFA) = 95:5): $t_R(R)$ = 81.8 min, $t_R(S)$ = 96.8 min.

5-Hydroxy-5-(3,5-dimethoxyphenyl)-4-oxopentanoic acid **(3k)**:



$C_{13}H_{16}O_6$, M_r 268.26; ^{1}H NMR ($CDCl_3$): δ = 2.52–2.60 (m, 2H), 2.65–2.79 (m, 2H), 3,78 (s, 1H), 5.07 (s, 1H), 6.43 (t, 1H, *J* = 2.2 Hz), 6.46 (d, 2H, *J* = 2.2 Hz) ppm; ^{13}C NMR ($CDCl_3$): δ = 27.5 (CH_2), 32.3 (CH_2), 55.4 (CH_3), 79.7 (CH), 100.8 (CH), 105.3 (CH), 139.9 (C), 161.3 (C), 177.1 (COOH), 207.5 (C=O) ppm; GC-MS (EI): t_R = 11.4 min, *m/z* (%) 222 (12) $[M - CO_2 - 2H]^+$, 165 (100) $[C_9H_9O_3]^+$, 137 (25) $[C_8H_9O_2]^+$, 122 (23) $[C_7H_6O_2]^+$, 107 (6) $[C_6H_3O_2]^+$, 92 (1) $[C_6H_4O]^+$, 77 (5) $[C_6H_5]^+$; $t_{R(lactone)}$ = 14.0 min, *m/z* (%) 250 (15) $[M]^+$, 165 (100) $[C_9H_9O_3]^+$, 137 (19) $[C_8H_9O_2]^+$, 122 (12) $[C_7H_6O_2]^+$, 107 (6) $[C_6H_3O_2]^+$, 85 (6) $[C_4H_5O_2]^+$, 77 (5) $[C_6H_5]^+$; HPLC-DAD: (Daicel Chiralpac IC 5 µm, 20 ºC, 1.2 mL min^{-1}: *n*-hexane/2-propanol (0.25% TFA) = 85:15): $t_R(R)$ = 18.0 min, $t_R(S)$ = 25.6 min.

7. References

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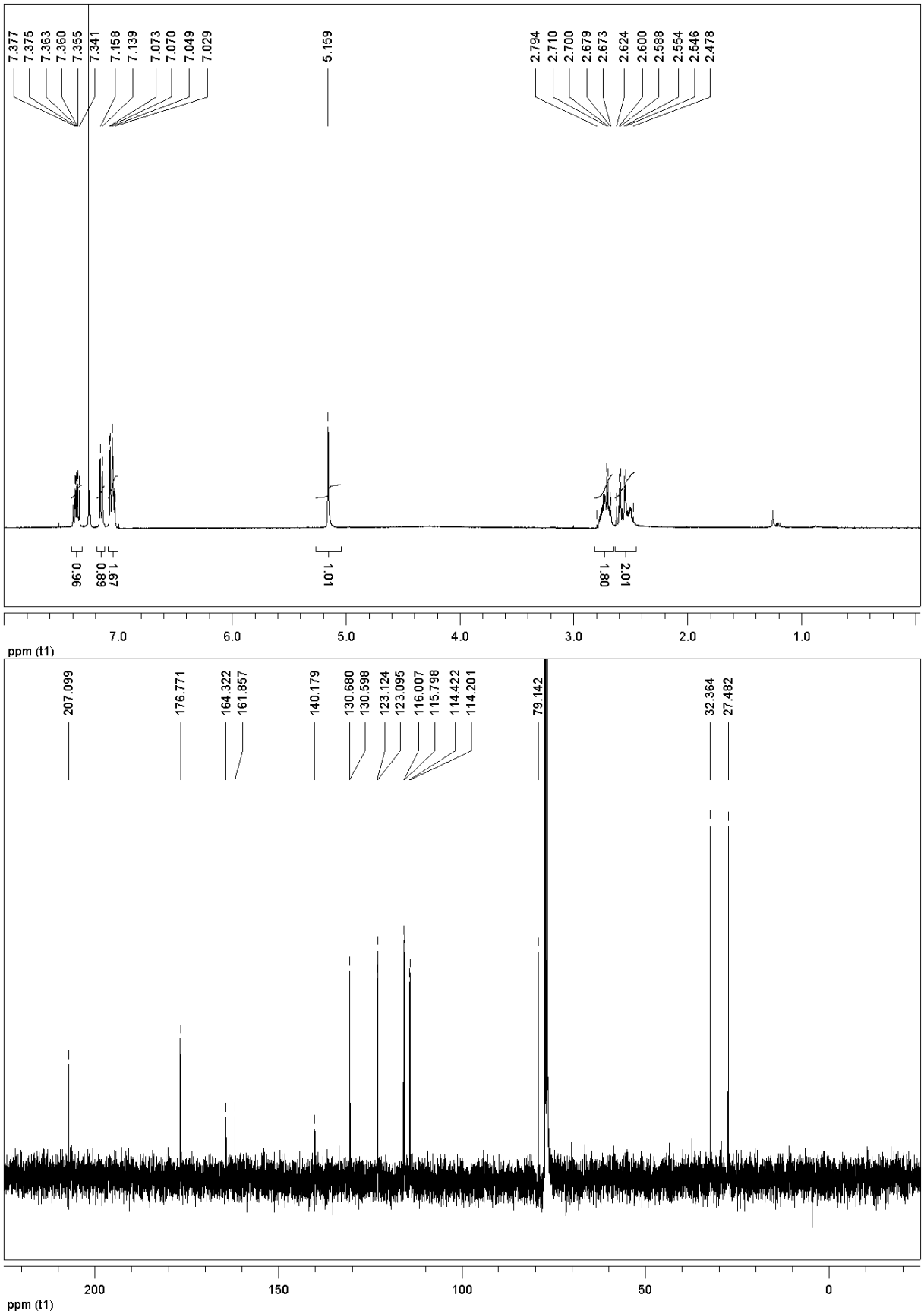
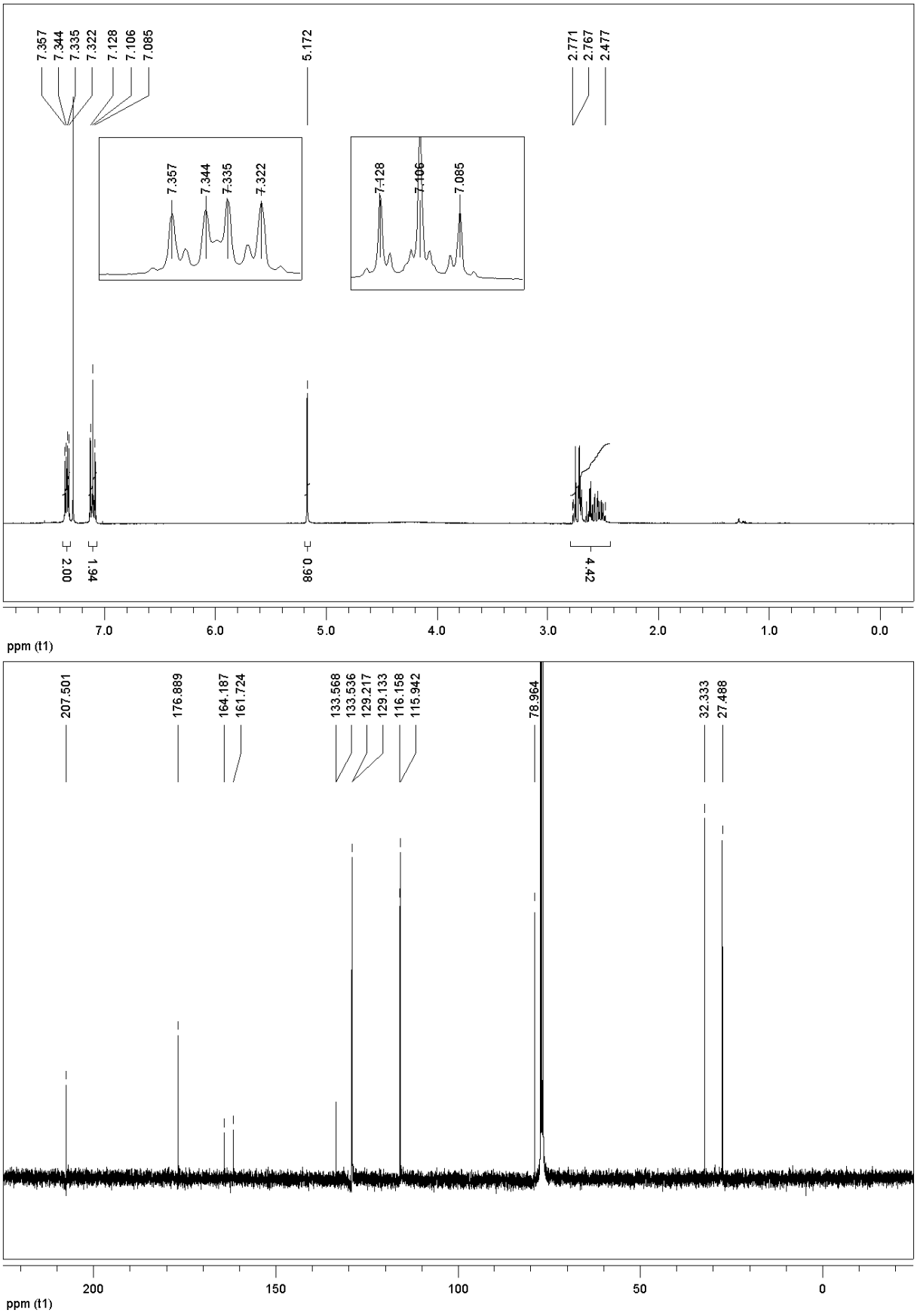
[7] M. Beigi, S. Waltzer, L. Eggeling, G. A. Sprenger and M. Müller, Org. Lett., 2012, *in press*, *DOI* 10.1021/ol3031186.

8. ^{1}H and ^{13}C NMR spectra

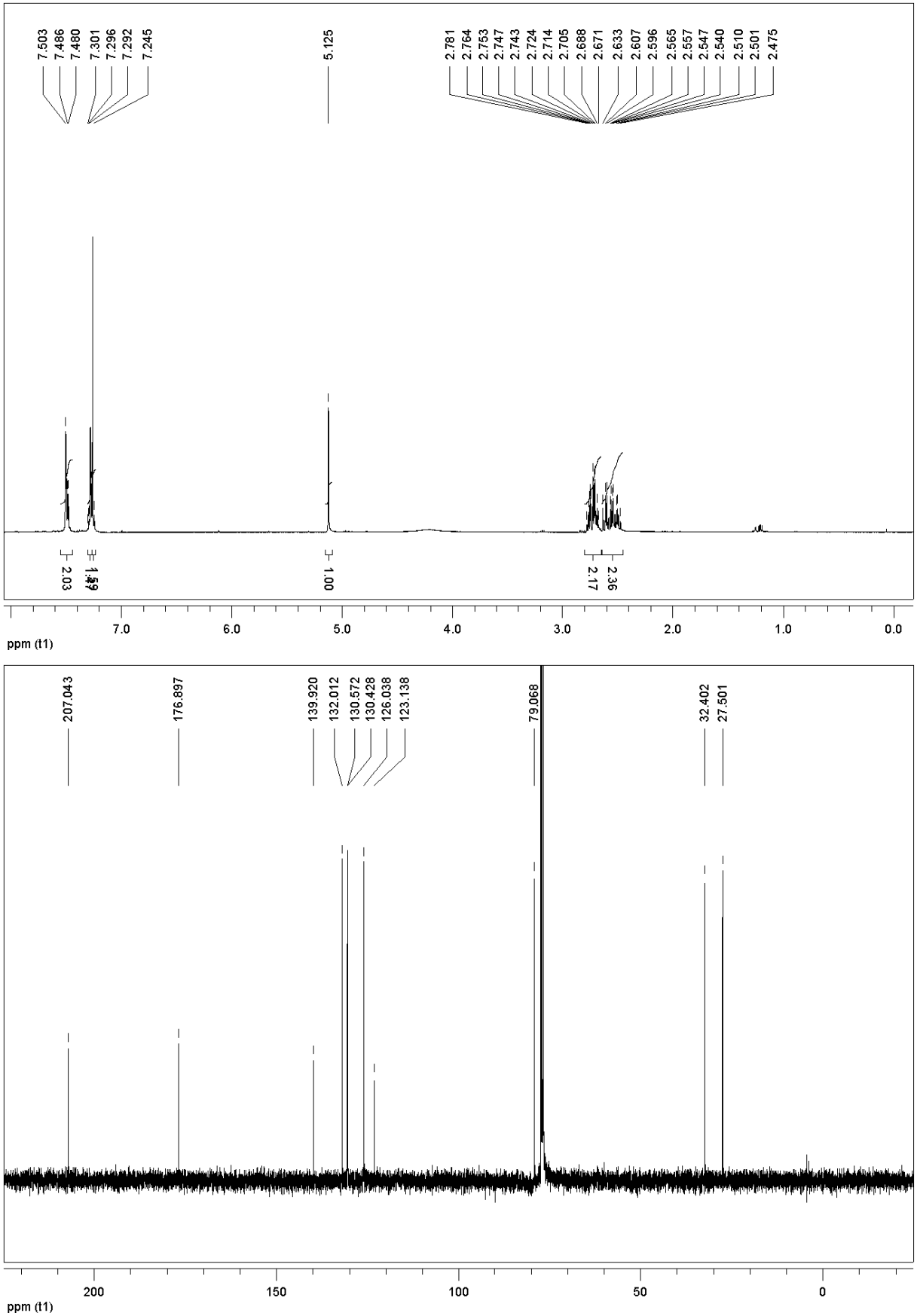
^{1}H and ^{13}C NMR spectrum of 5-(3-Fluorophenyl)-5-hydroxy-4-oxopentanoic acid (**3c**):



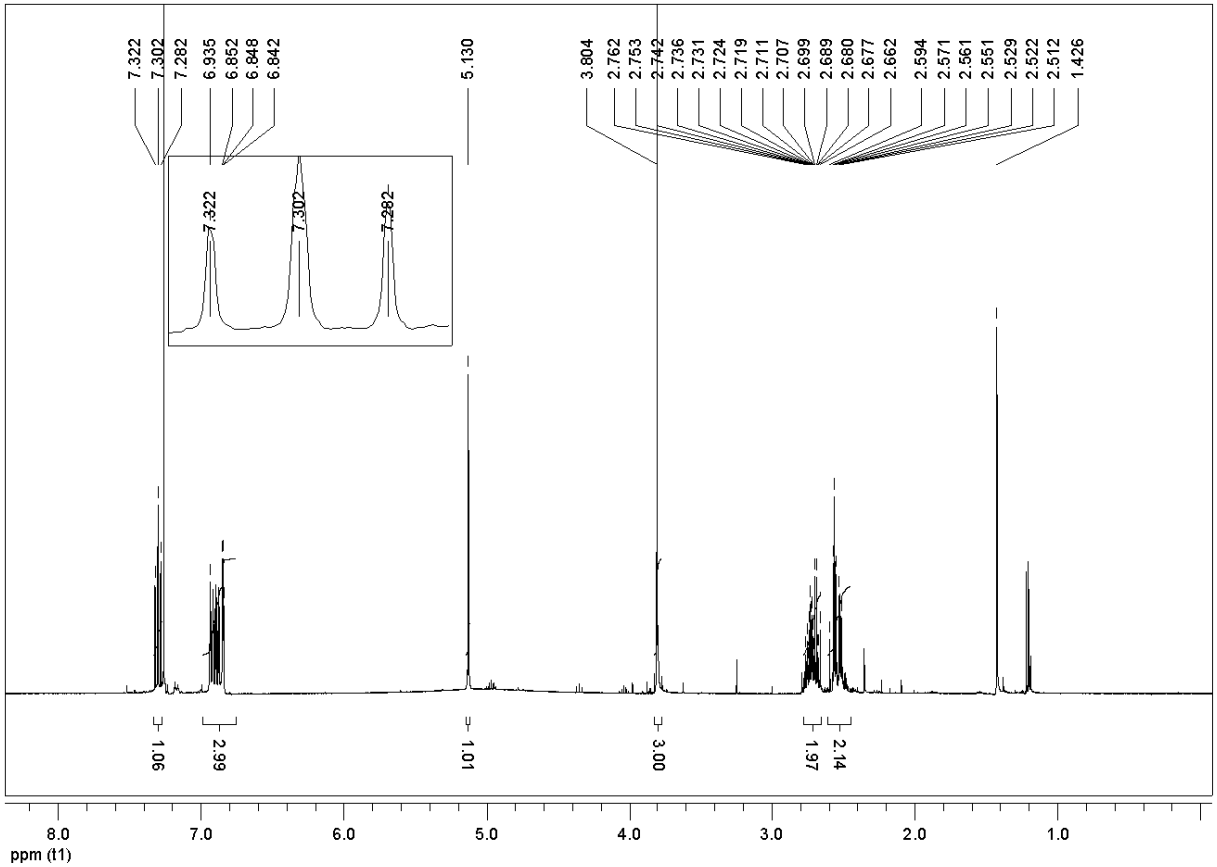
^{1}H and ^{13}C NMR spectrum of 5-(4-Fluorophenyl)-5-hydroxy-4-oxopentanoic acid (**3d**):

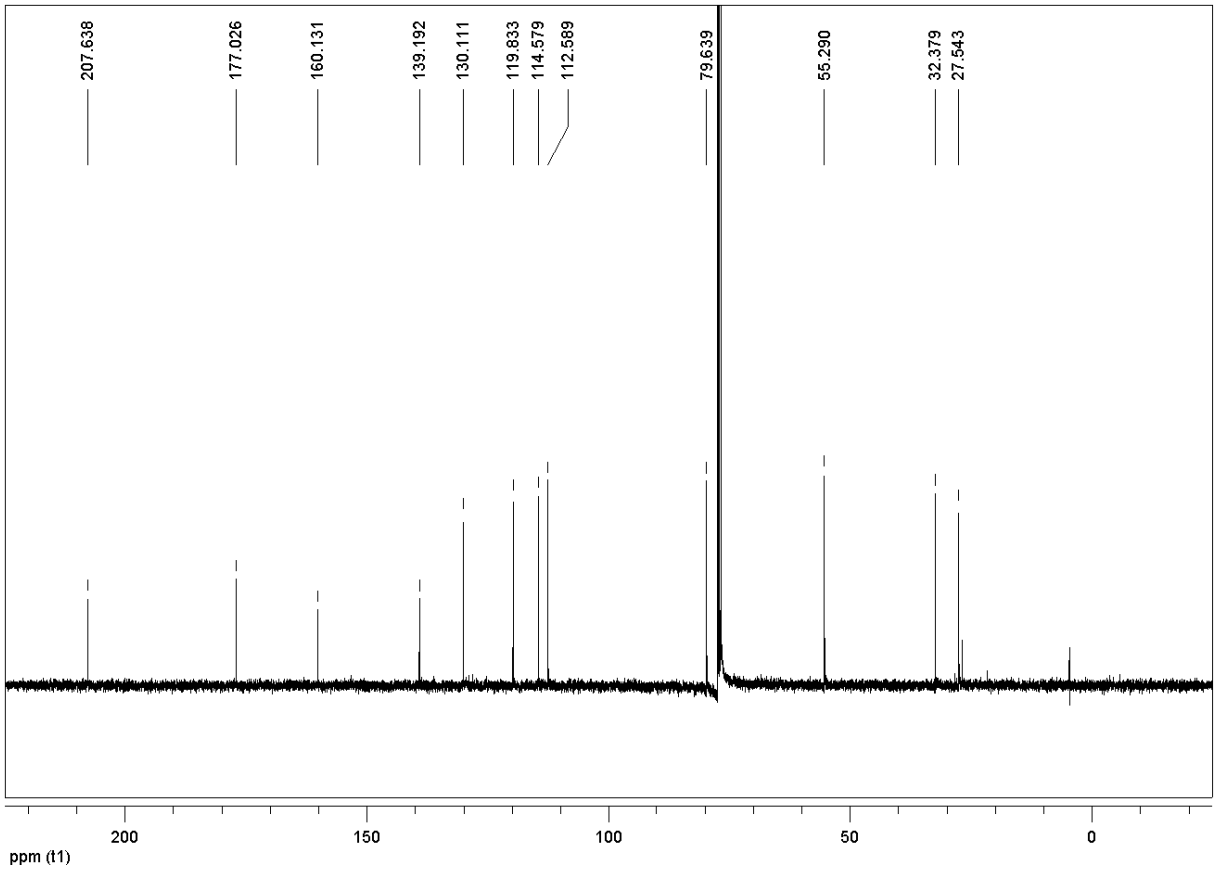


^{1}H and ^{13}C NMR spectrum of 5-(3-Bromophenyl)-5-hydroxy-4-oxopentanoic acid (**3h**):

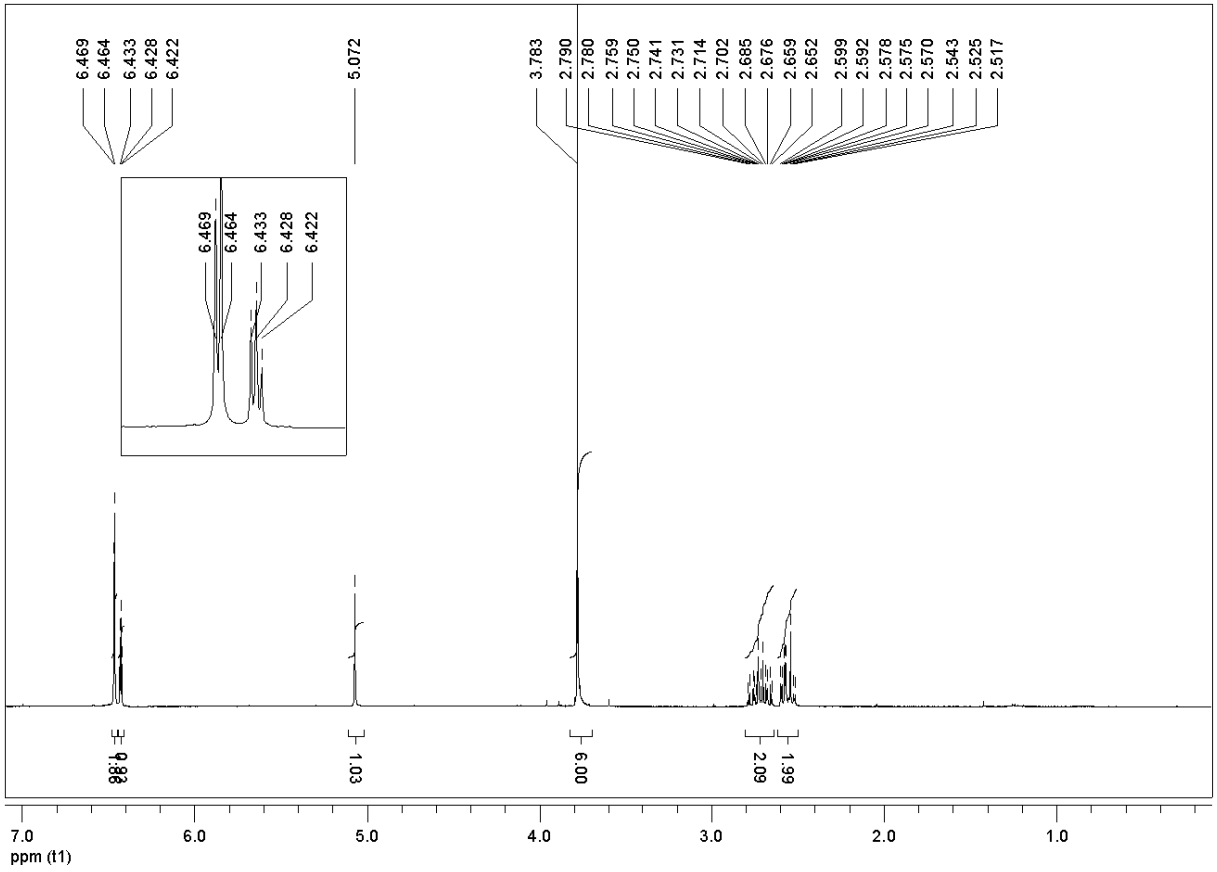


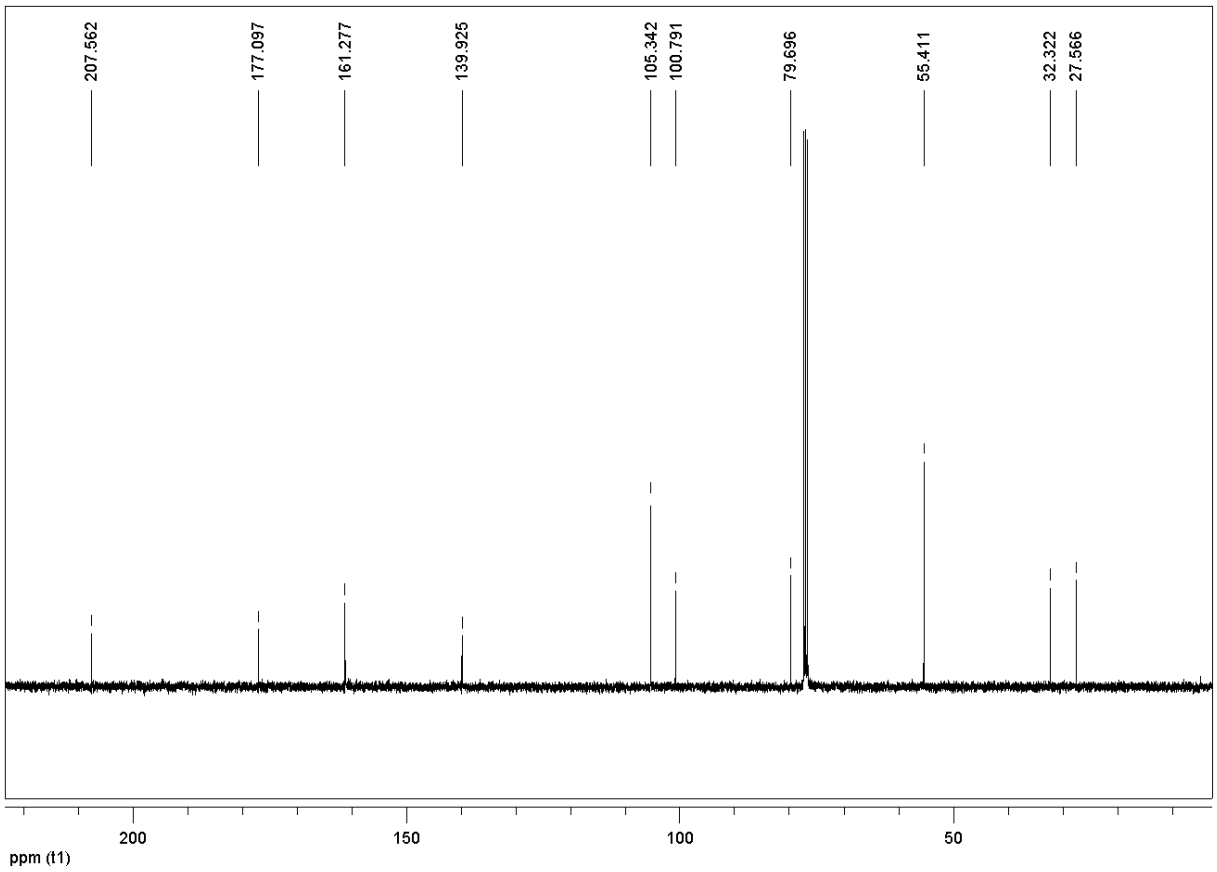
^{1}H and ^{13}C NMR spectrum of 5-Hydroxy-5-(3-methoxyphenyl)-4-oxopentanoic acid (**3j**):





^{1}H and ^{13}C NMR spectrum of 5-Hydroxy-5-(3,5-dimethoxyphenyl)-4-oxopentanoic acid (**3k**):

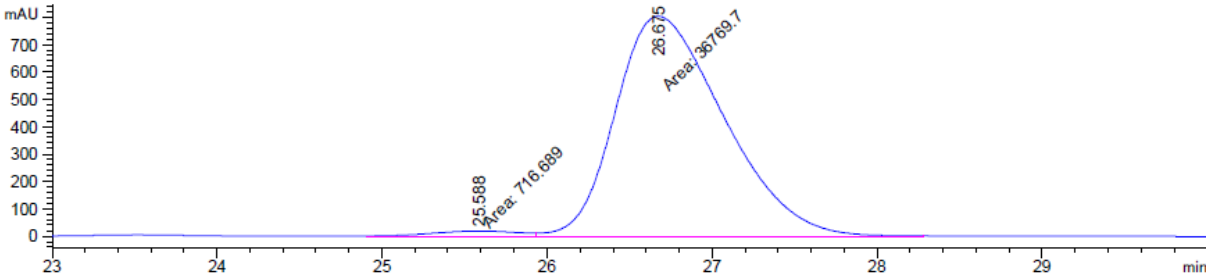




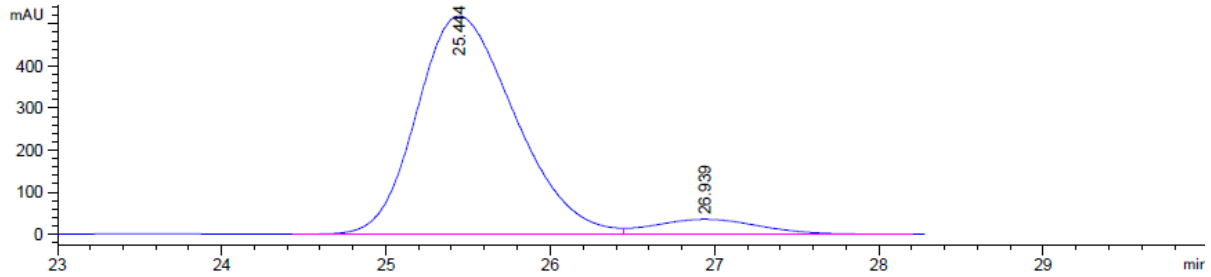
9. Chiral phase HPLC

HPLC of (*R*)- and (*S*)-5-(3-Fluorophenyl)-5-hydroxy-4-oxopentanoic acid (**3c**):

EcMenDwt: ee = 96% (R)

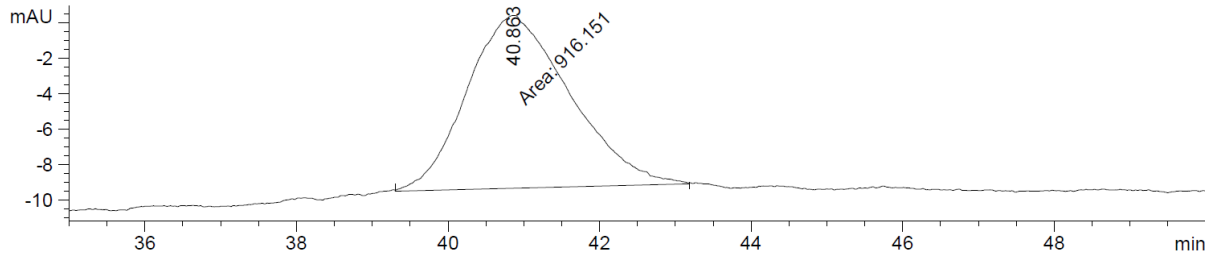


EcMenDI474A/F475G: ee = 82% (S)

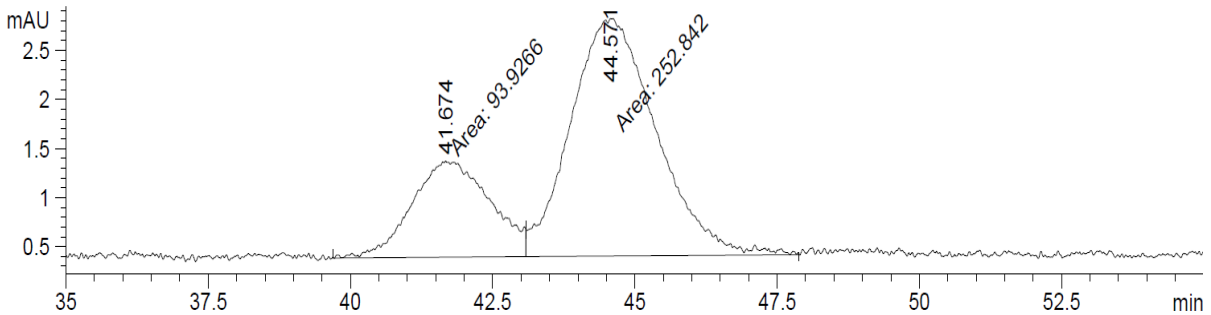


HPLC of (*R*)- and (*S*)-5-(4-Fluorophenyl)-5-hydroxy-4-oxopentanoic acid (**3d**):

EcMenDwt: ee = 99% (R)

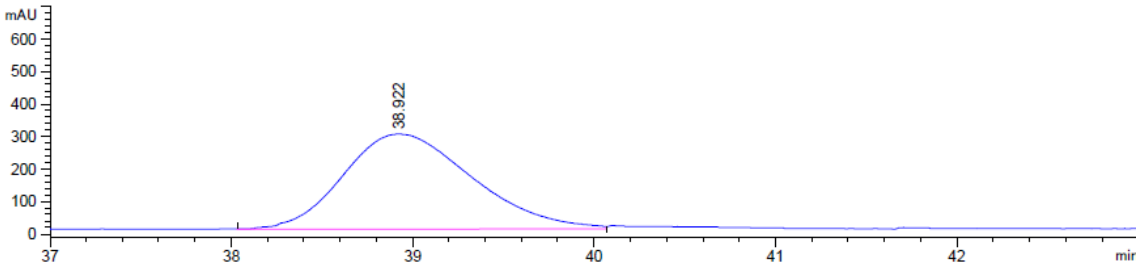


EcMenDI474A/F475G: ee = 46% (S)

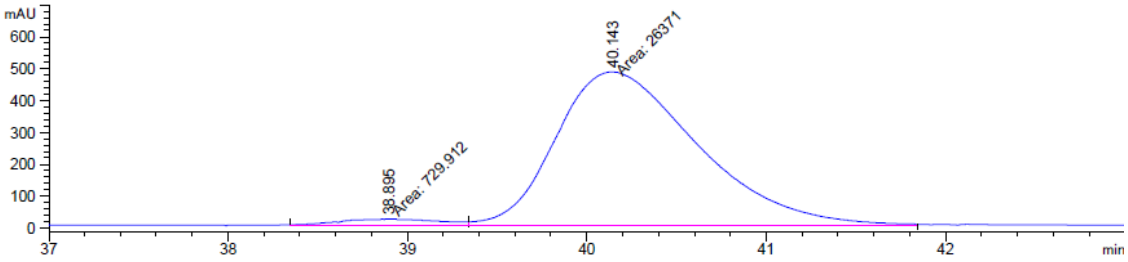


HPLC of (*R*)- and (*S*)-5-(3-Bromophenyl)-5-hydroxy-4-oxopentanoic acid (**3h**):

EcMenDwt: ee = 99% (R)

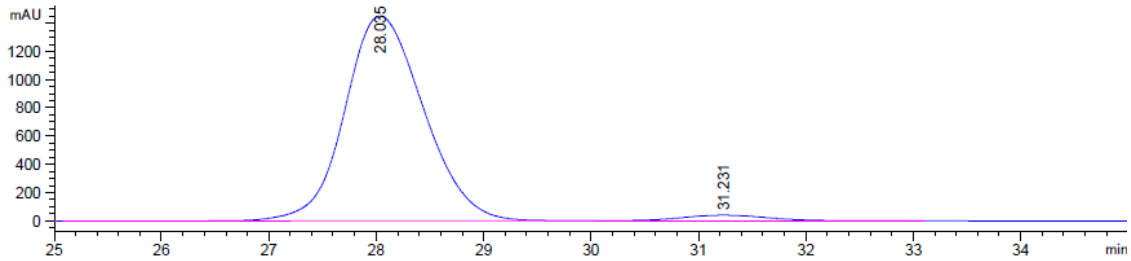


EcMenDI474A/F475A: ee = 95% (S)

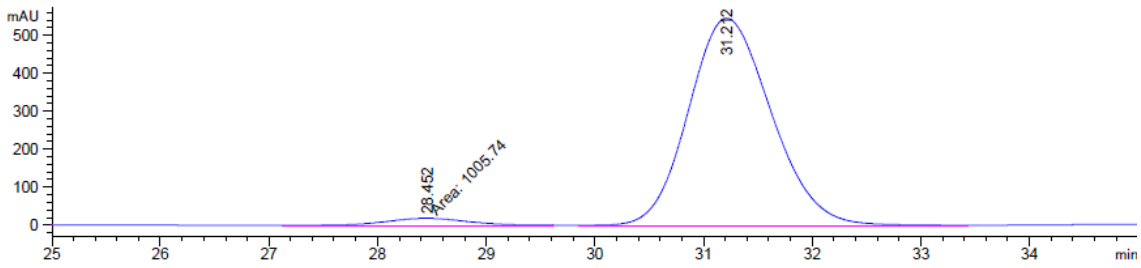


HPLC of (*R*) and (*S*)-5-Hydroxy-5-(3-iodophenyl)-4-oxopentanoic acid (**3i**):

EcMenDwt: ee = 94% (R)

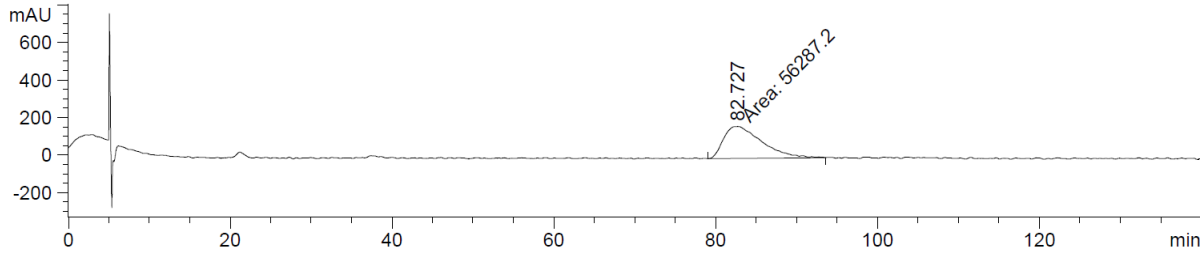


EcMenDI474A/F475G: ee = 93% (S)

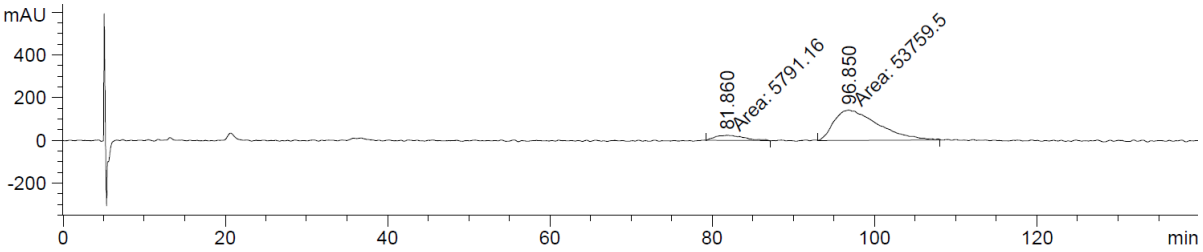


HPLC of (*R*) and (*S*)-5-Hydroxy-5-(3-methoxyphenyl)-4-oxopentanoic acid (**3j**):

EcMenDwt: ee = 99% (R)

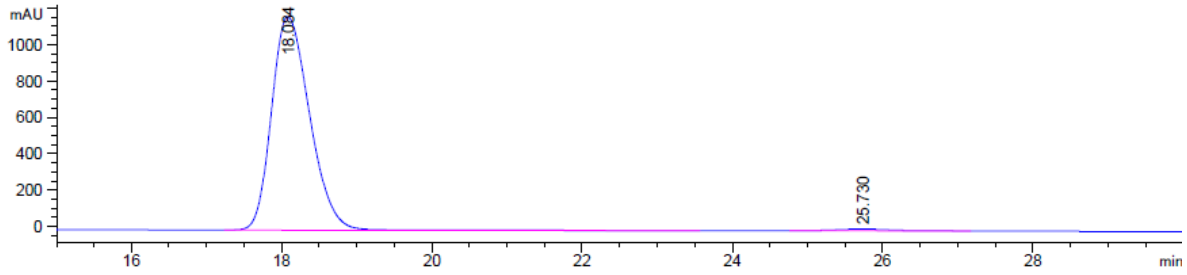


EcMenDI474G/F475G: ee = 78% (S)

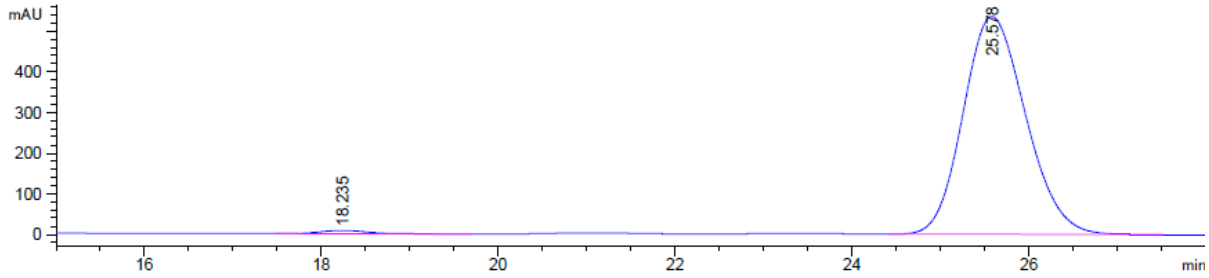


HPLC of (*R*)- and (*S*)-5-Hydroxy-5-(3,5-dimethoxyphenyl)-4-oxopentanoic acid (**3k**):

EcMenDwt: ee = 98% (R)



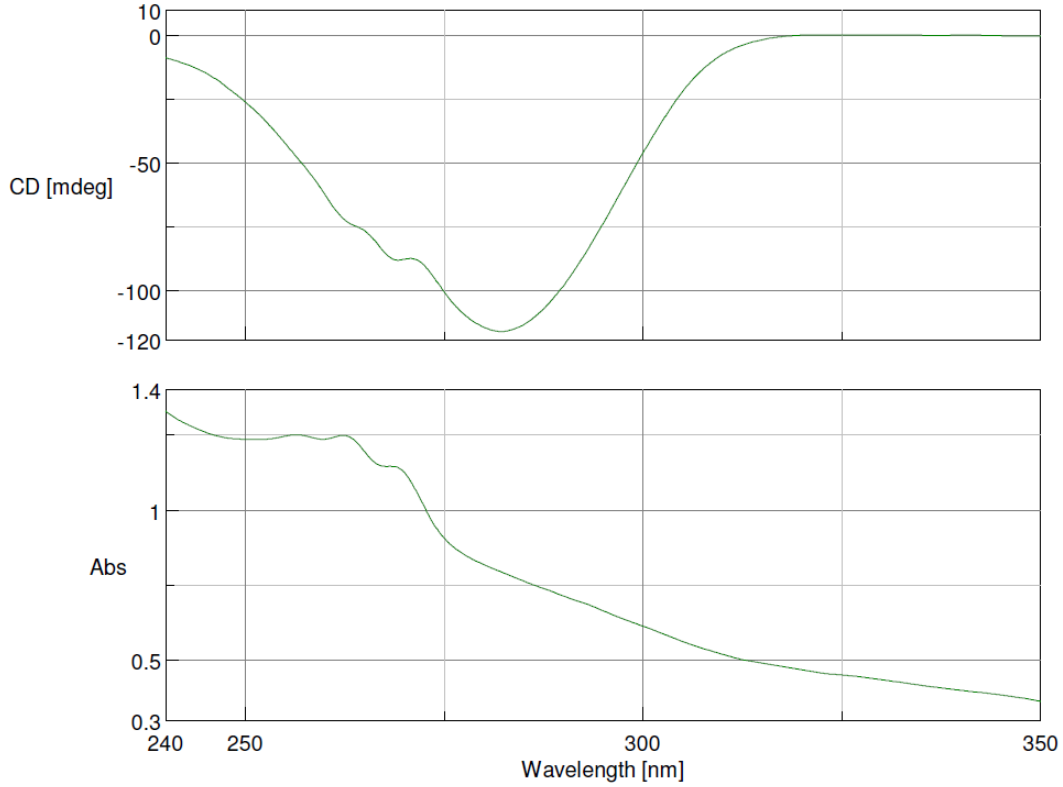
EcMenDI474A/F475G: ee = 96% (S)



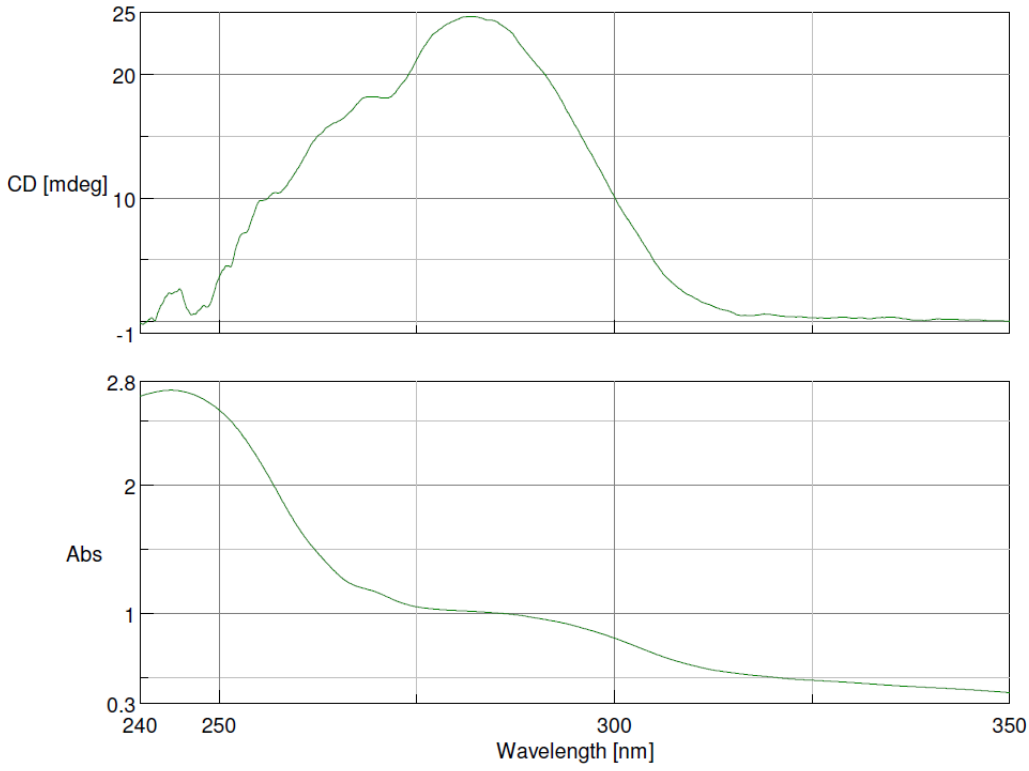
10. CD spectra

CD spectrum of (*R*)- and (*S*)-5-(3-Fluorophenyl)-5-hydroxy-4-oxopentanoic acid (**3c**):

EcMenDwt: ee = 96% (R)

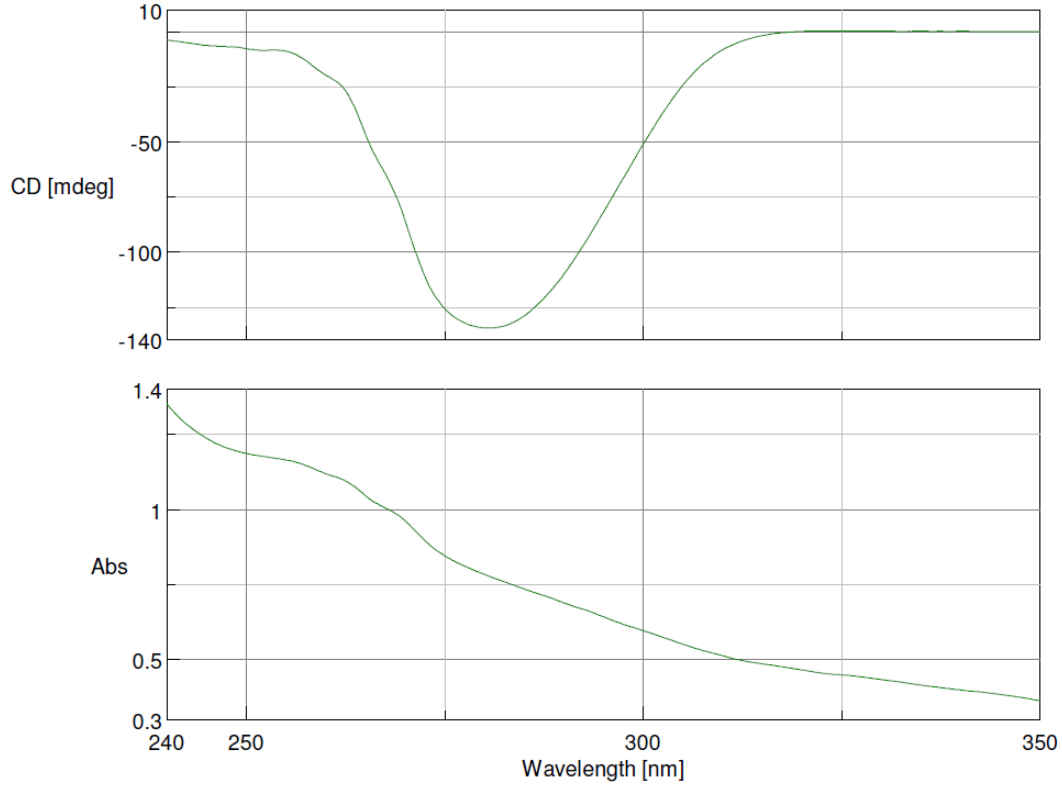


EcMenDI474G/F475G: ee = 59% (S)

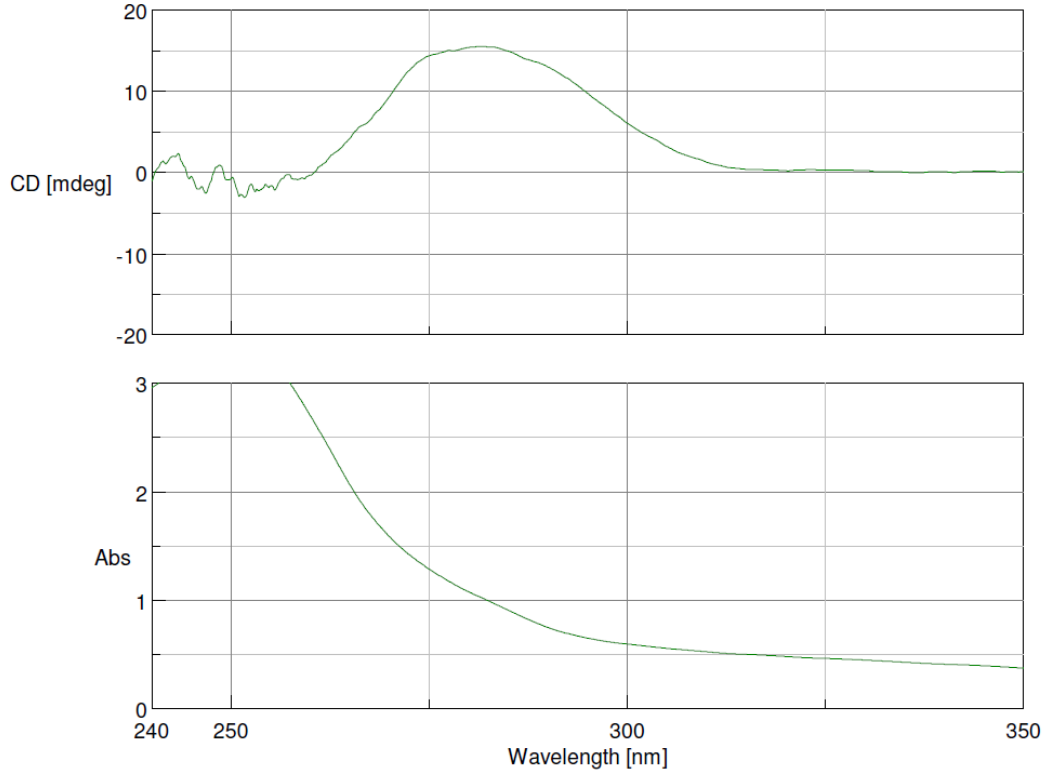


CD spectrum of (*R*)- and (*S*)-5-(4-Fluorophenyl)-5-hydroxy-4-oxopentanoic acid (**3d**):

EcMenDwt: ee = 99% (R)

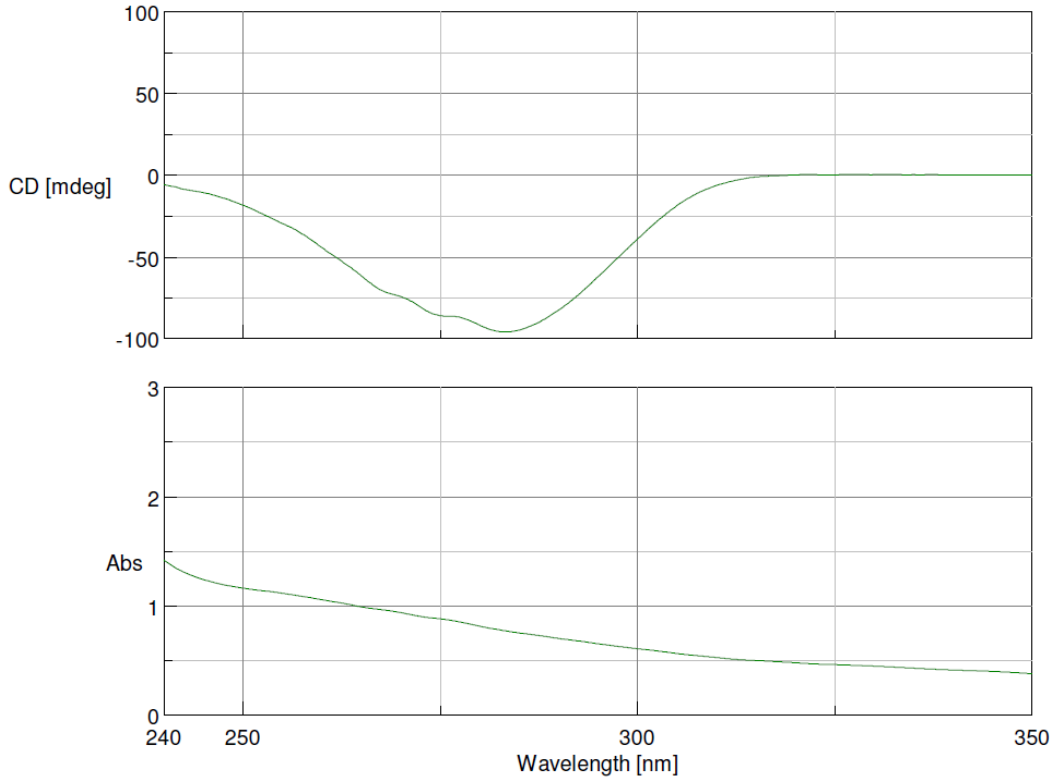


EcMenDI474A/F475A: ee = 30% (S)

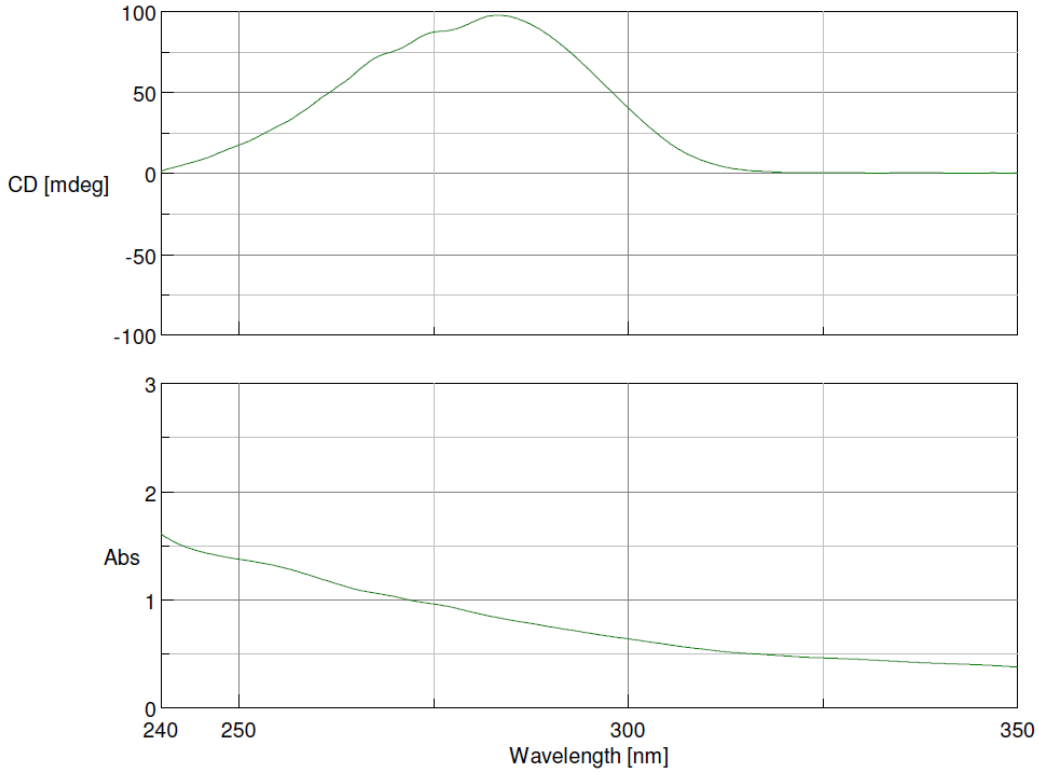


CD spectrum of (*R*)- and (*S*)-5-(3-Bromophenyl)-5-hydroxy-4-oxopentanoic acid (**3h**):

EcMenDwt: ee = 99% (R)

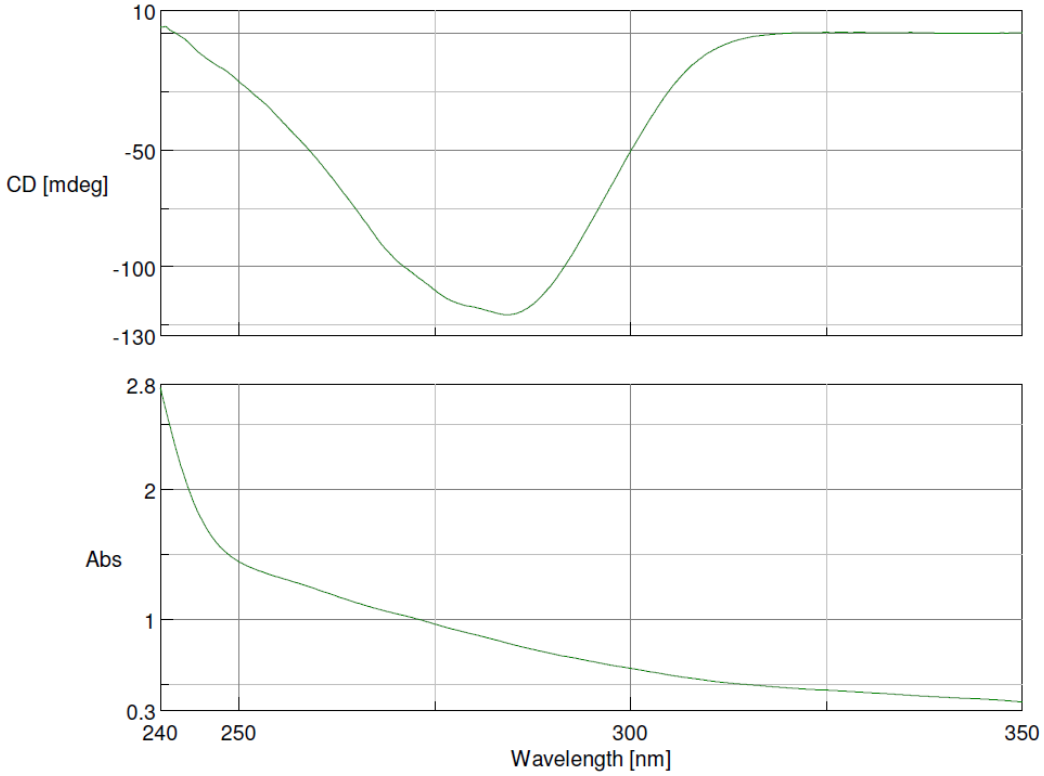


EcMenDI474G/F475G: ee = 94% (S)

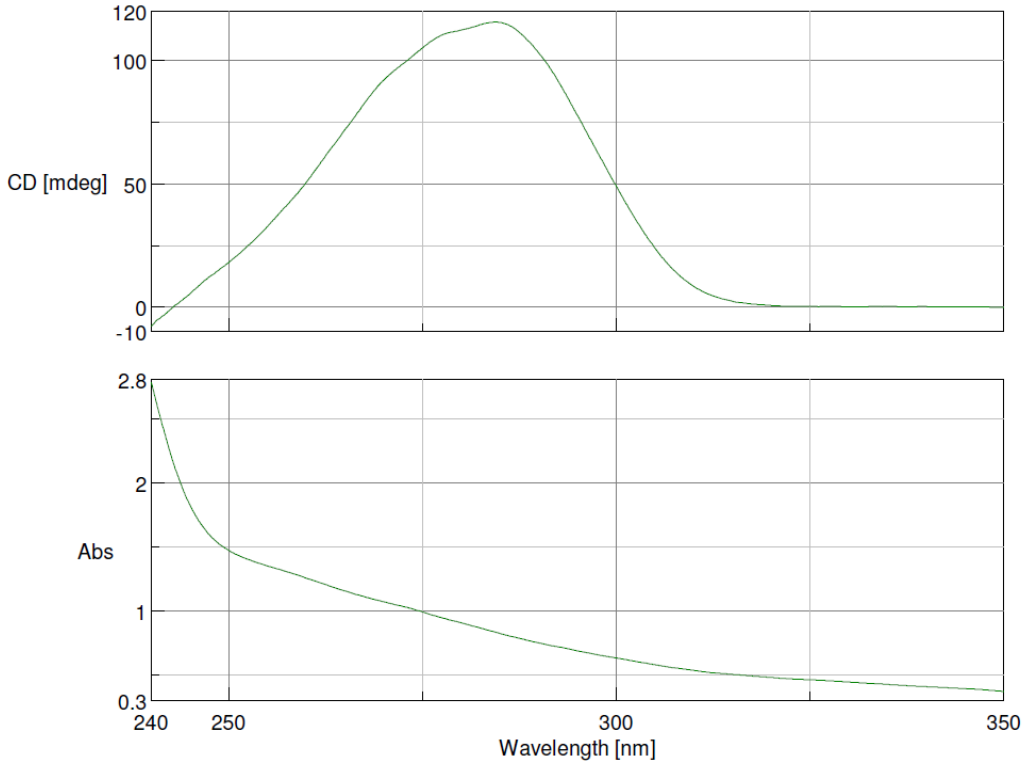


CD spectrum of (*R*)- and (*S*)-5-Hydroxy-5-(3-iodophenyl)-4-oxopentanoic acid (**3i**):

EcMenDwt: ee = 94% (R)

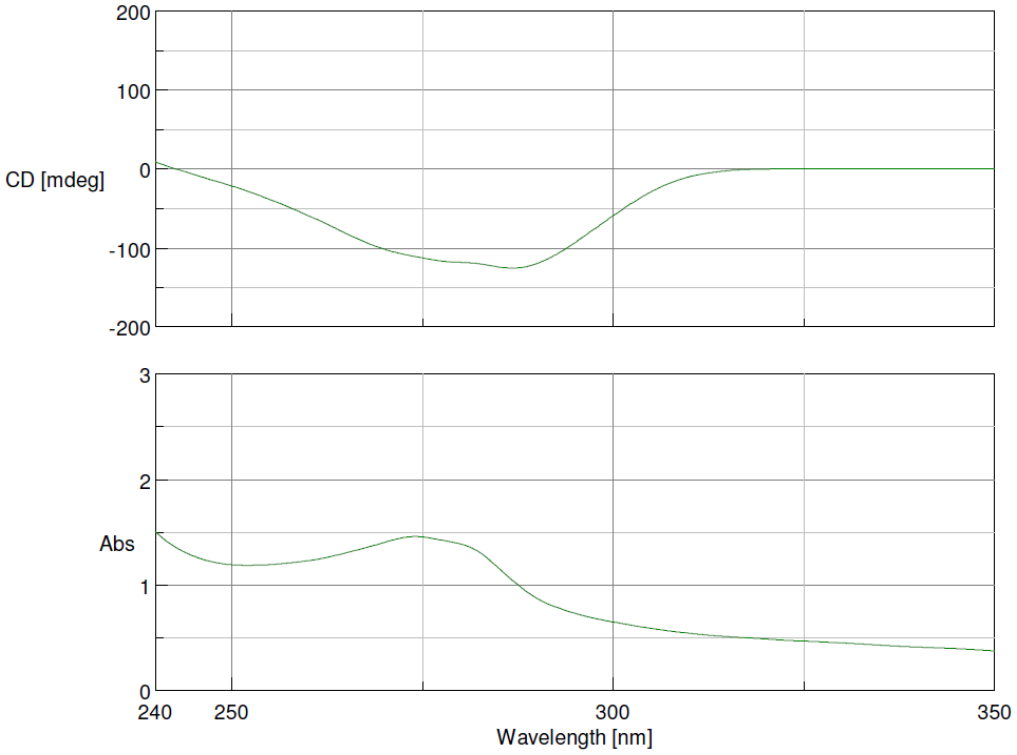


EcMenDI474G/F475G: ee = 91% (S)

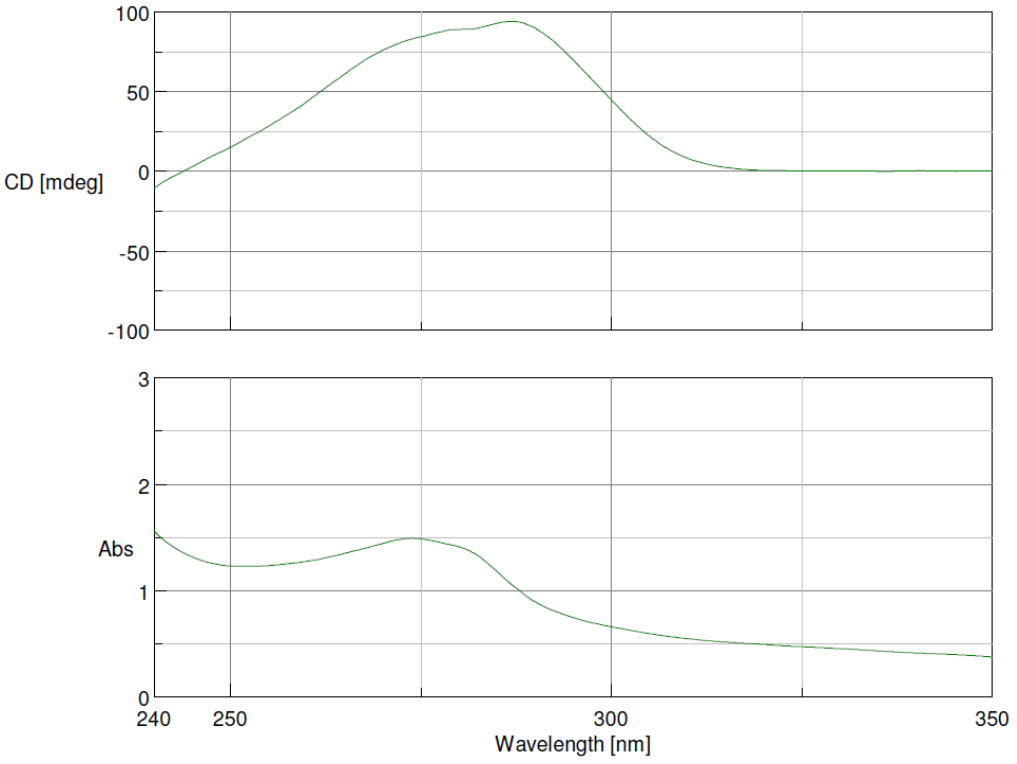


CD spectrum of (*R*)- and (*S*)-5-Hydroxy-5-(3-methoxyphenyl)-4-oxopentanoic acid (**3j**):

EcMenDwt: ee = 99% (R)

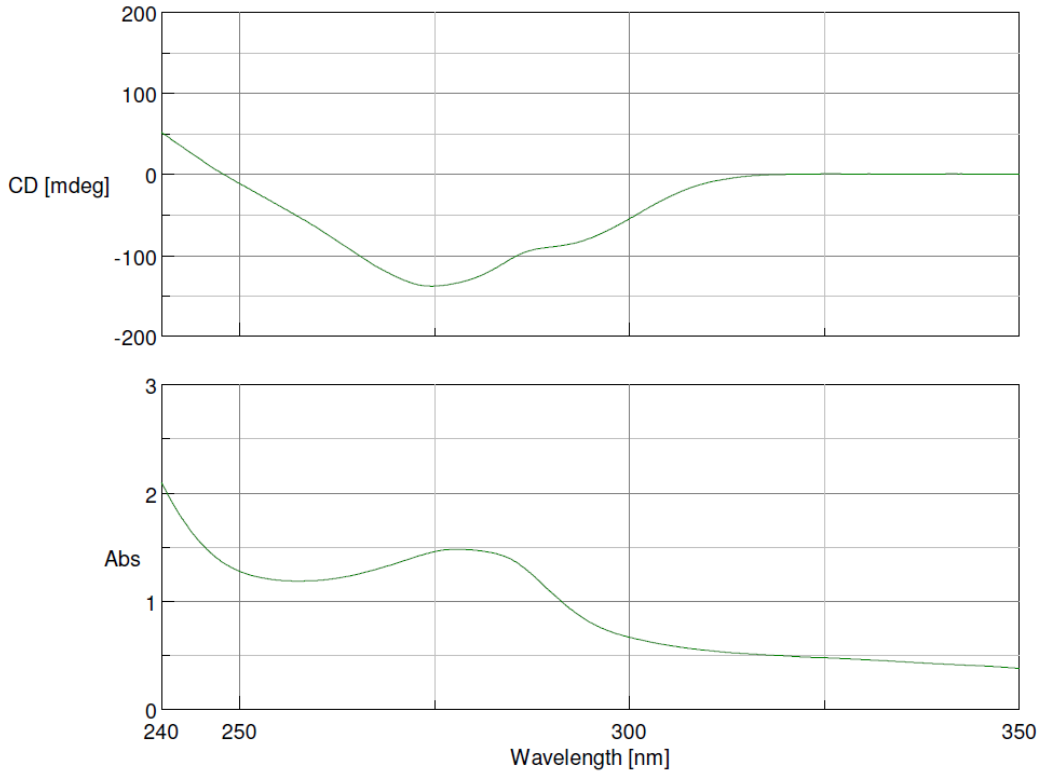


EcMenDI474G/F475G: ee = 78% (S)



CD spectrum of (*R*)- and (*S*)-5-Hydroxy-5-(3,5-dimethoxyphenyl)-4-oxopentanoic acid (**3k**):

EcMenDwt: ee = 98% (R)



EcMenDI474G/F475G: ee = 85% (S)