Electronic Supplementary Information

For

Stereoselective synthesis of γ-hydroxy-α-amino acids through Aldolase-Transaminase recycling cascades

Christine Guérard-Hélaine, Egon Heuson, Moussa Ndiaye, Léa Gourbeyre, Marielle Lemaire, Virgil Hélaine, Franck Charmantray, Jean-Louis Petit, Marcel Salanoubat, Véronique de Berardinis and Thierry Gefflaut*

Corresponding author : Thierry Gefflaut@univ-bpclermont.fr

Content

1. General Informations

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer. Chemical shifts are reported in ppm (δ) relative to TMS as internal standard. HRMS were recorded on a q-tof Micromass spectrometer. Optical rotations were determined with a JASCO DIP 370 polarimeter and are reported at the sodium D line (589 nm). Precoated F_{254} plates were used for TLC chromatography with n-PrOH/H_2O (7:3) as eluent and 2 g . L^{-1} ninhydrin in EtOH as revelator. Bovine heart malic dehydrogenase (MDH, EC: 1.4.1.3) and rabbit muscle lactic dehydrogenase (LDH, EC: 1.1.1.27) were purchased from Sigma. Ni-NTA agarose for IMAC chromatography was purchased from QIAGEN. PD-10 desalting columns were purchased from GE-Healthcare. One Unit (U) corresponds to the quantity of enzyme allowing the conversion of 1 µmol of substrate per minute in the specified conditions.

2. Synthesis of 4-hydroxy-2-oxoglutaric acid (HKG)

HKG was prepared following described procedures[1,2] with some modifications: Oxaloacetic acid (0.5 g, 3.78 mmol) and glyoxylic acid (250 mg, 3.38 mmol) were dissolved in 1 M NaOH (9 mL, 9 mmol) and pH was rapidly adjusted to 7.8 with 1 M NaOH (approx. 2 mL). The solution was stirred at room temperature for 4 h. pH was then adjusted to 3 by addition of Dowex® 50WX8 (H^+ form) and the mixture stirred for 30 min. After filtration, pH was adjusted to 7 with 4 M and 1 M NaOH and the volume was finally adjusted to 15 mL with H_2O. HKG and Pyruvic acid (PA) titrations were achieved as described in section 8 indicating concentrations of 205 and 7 mM for HKG and PA respectively. An aliquot of the solution (1 mL) was lyophilised for further analyses. ^{1}H NMR (400 MHz, D_2O) δ 4.40 (1H, dd, *J* = 3.0 and 8.5 Hz, H^4), 3.23 (1H, dd, *J* = 3.0 and 18.0 Hz, H^{3a}), 3.09 (1H, dd, *J* = 8.5 and 18.0 Hz, H^{3b}); ^{13}C NMR (100 MHz, D_2O) δ 203.1 (C^2), 180.2 (C^5), 169.1 (C^1), 67.7 (C^4), 44.2 (C^3); HRMS (ES-) *m/z* 161.0080 ([M-H]$^-$, calc. for $C_5H_5O_6$: 161.0081).

3. Synthesis of 4,5-dihydroxy-2-oxopentanoic acid (DHOP)

A mixture of 1 M sodium pyruvate (1 mL, 1 mmol), 1 M Glycolaldehyde (1.1 mL, 1.1 mmol), 0.1 M $MgCl_2$ (0.1 mL, 10 µmol) and H_2O (4 mL) was adjusted to pH 7.6 with 1M NaOH. The solution volume was adjusted to 10 mL with H_2O and 10 mg AL2 (uniprot id. B7NJZ1) were added. The solution was gently stirred at 20 °C for 24 h. It was then passed through a short column of Ni-NTA Agarose (2 mL) to remove the tagged-enzyme and lyophilized. DHOP was isolated as a white solid (205 mg, quant.). NMR analyses indicated a conversion of 96% and that DHOP exists in aqueous solution as a mixture of 1 open (30%) and 2 cyclic hemiketal forms (39 and 31%). ^{1}H NMR (400 MHz, D_2O) open form: δ 4.18 (1H, m, H^4), 3.58 (1H, dd, *J* = 4.0 and 12.0 Hz, H^{5a}), 3.51 (1H, dd, *J* = 7.0 and 12.0 Hz, H^{5b}), 2.95 (1H, dd, *J* = 4.5 and 17.0 Hz, H^{3a}), 2.88 (1H, dd, *J* = 8.0 and 17.0 Hz, H^{3b}), major cyclic form: δ 4.54 (1H, m, H^4), 4.11 (1H, dd, *J* = 4.5 and 10.0 Hz, H^{5a}), 3.88 (1H, ddd, *J* = 1.0, 2.0 and 10.0 Hz, H^{5b}), 2.33-2.22 (1H, m, H^3), minor cyclic form: δ 4.54 (1H, m, H^4), 4.08 (1H, dd, *J* = 4.5 and 13.0 Hz, H^{5a}), 3.99 (1H, ddd, *J* = 1.0, 2.0 and 10.0 Hz, H^{5b}), 2.42 (1H, dd, *J* = 6.0 and 14.0 Hz, H^{2a}), 2.04 (1H, d, *J* = 14.0 Hz, H^{2b}); ^{13}C NMR (100 MHz, D_2O) open form: δ 203.7 (C^2), 169.4 (C^1), 67.4 (C^4), 64.9 (C^5), 42.8 (C^3), major cyclic form: δ 176.9 (C^1), 103.8 (C^2), 74.9 (C^5), 71.2 (C^4), 44.2 (C^3), minor cyclic form: δ 177.0 (C^1), 103.9 (C^2), 75.6 (C^5), 70.5 (C^4), 43.9 (C^3); HRMS (ES-) *m/z* 147.0287 ([M-H]$^-$, calc. for $C_5H_7O_5$: 147.0288).

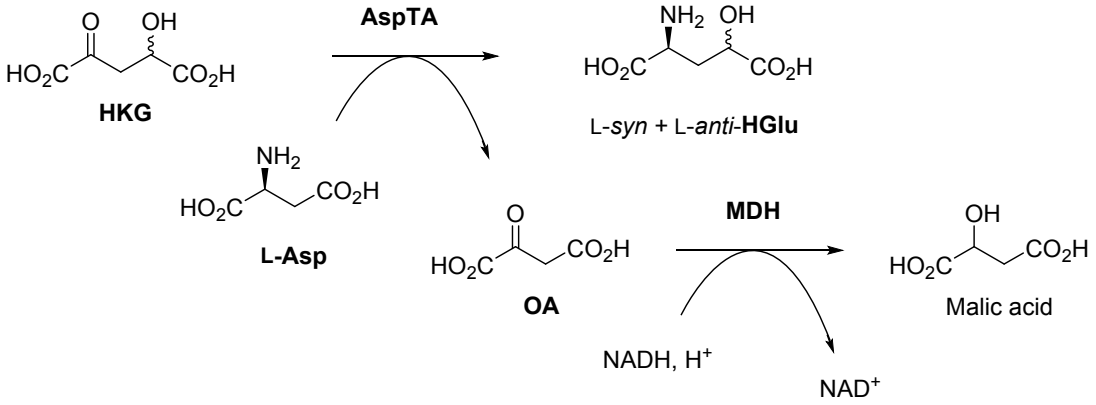
4. Enzyme assays

Enzyme assays were run at 25 °C in disposable 1 mL cuvettes using a UV/Vis spectrometer (Agilent Cary™ 300) or at 30 °C in Greiner® 96-well plates using a multimode microplate reader (Safire™ II, Tecan). All measurements were performed in 50 mM potassium phosphate (KP) buffer (pH 7.5) in a total volume of 1 mL (cuvette) or 200 µL (microplate). In

the latter case, an optical path of 0.59 cm was determined. Absorbance variations were recorded at 340 nm and initial rates were calculated from slopes using ε = 6220 $M^{-1}.cm^{-1}$ for NADH.

4.1 AspTA assay with HKG

AspTA activity was measured at 30 °C in microplates in a total volume of 0.2 mL using the assay described in scheme S1.

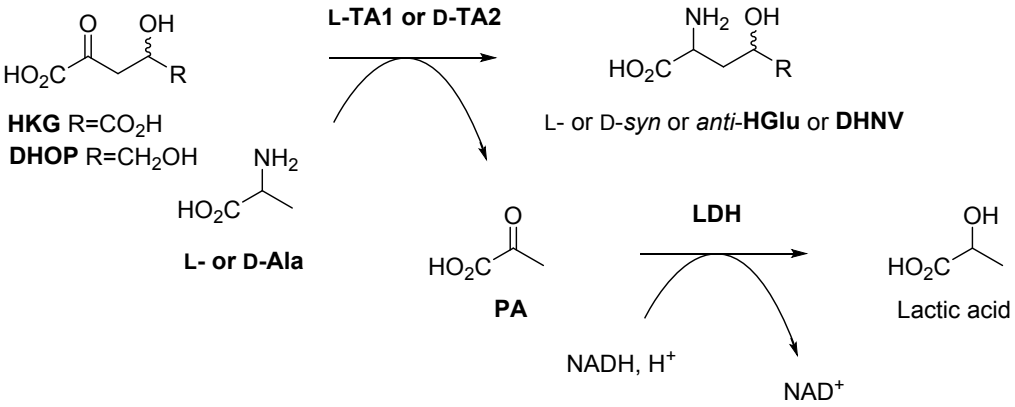


Scheme S1 AspTA assay with Asp as amino donor substrate.

Assay solutions contained AspTA (≤ 50 mU), HKG (0.1-10 mM), NADH (0.8 mM), MDH (2U), Asp (20 mM).

4.2 Assays for L-TA1 (uniprot Id. G0VQA2) and D-TA2 (uniprot Id. E6LHY8) with HKG and DHOP

Activities of both TA were measured at 30 °C in microplates in a total volume of 0.2 mL using the assay described in scheme S2.



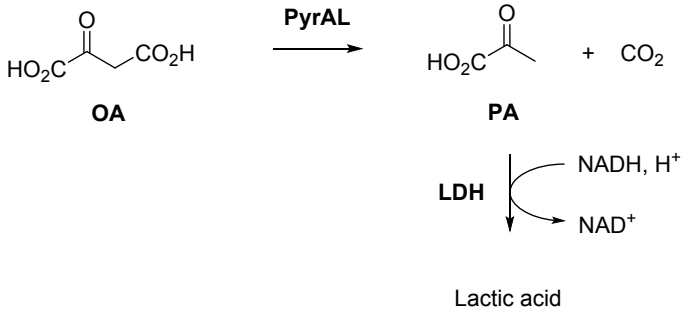
Scheme S2 L- or D-TA assay with Ala as amino donor substrate.

Assay solutions contained L- or D-TA (≤ 50 mU), Pyridoxal phosphate (0.01 mM for L-TA1, 0.1 mM for D-TA2), HKG or DHOP (0.1-10 mM), NADH (0.8 mM), LDH (2U), L- or D-Ala (20 mM (with HKG) or 100 mM (with DHOP)).

4.3 Pyruvate-aldolase assays

4.3.1 OA decarboxylation

Decarboxylation activity of PyrALs was measured at 25 °C in 1 mL total volume using te assay described in scheme S3.

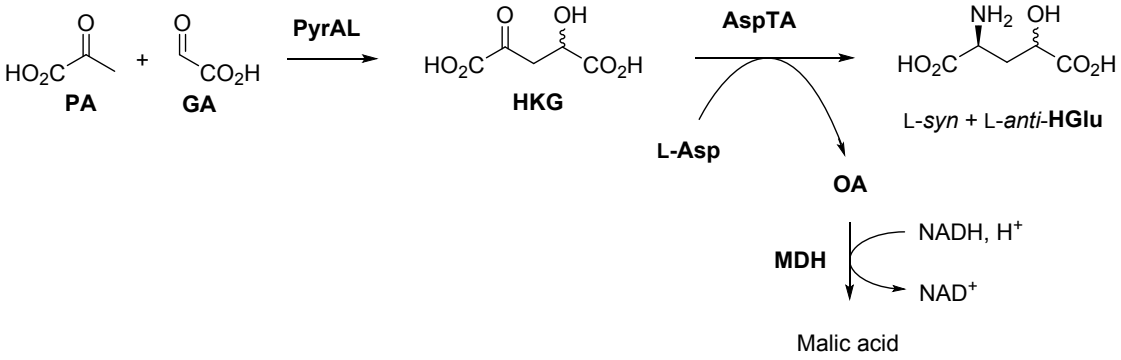


Scheme S3 OA decarboxylation assay

Assay solutions contained PyrAL (≤ 25 mU), OA (0.5 mM), NADH (0.4 mM), LDH (1U), $MnCl_2$ (0.5 mM). Reactions were initiated by addition of OA after equilibration of the system for 5 minutes at 25 °C. A control experiment was systematically performed in the same conditions but without PyrAL and the control slope was substracted from the assay slope.

4.3.2 Aldolisation with PA and GA

Aldolisation activity of PyrALs were measured at 25 °C in 1 mL total volume using the assay described in scheme S4.



Scheme S4 Aldolisation assay

Assay solutions contained PyrAL (≤ 25 mU), PA (1-50 mM), GA (1-50 mM), NADH (0.4 mM), AspTA (0.5 U), MDH (2U), L- Asp (40 mM), $MnCl_2$ (0.5 mM). Reactions were initiated by addition of GA after equilibration of the system for 5 minutes at 25 °C. A control experiment was systematically performed in the same conditions but without PyrAL and the control slope was substracted from the assay slope.

5. Screening of Pyruvate aldolases

The assay described in section 4.3.2 was used with 10 mM PA and GA to estimate activities (U. mL^{-1}) in cell lysates containing 19 His-tagged PyrAL. A series of small scale syntheses of HGlu was then carried out in a total volume of 10 mL containing PA (10 mM), GA (10 mM), AspTA (10 U), PyrAL (1U, 22-165 µL cell lysate), Asp (10 mM), $MnCl_2$ (1 mM) and potassium phosphate 20 mM, pH 7,6. The reaction mixture was stirred at room temperature for 24 h

and was then poured on a small column of Dowex®50Wx8 (H^+ form, 1 mL). The column was washed with H_2O (5 mL) and then eluted with 1 M NH_3. Ninhydrin-positive fractions were pooled and concentrated under reduced pressure. The residue was analysed by ^1H NMR to determine conversion and stereoselectivity. Conversion was calculated using the formula: conv. = ((*syn*-HGlu) + (*anti*-HGlu)) / ((*syn*-HGlu) + (*anti*-HGlu) + (Asp)). For example, Fig S1 shows the spectrum obtained with one PyrAL (Uniprot Id. Q2K7V6) indicating a conversion of 77% and a 45/55 mixture of *syn*- and *anti*-isomers of HGlu.

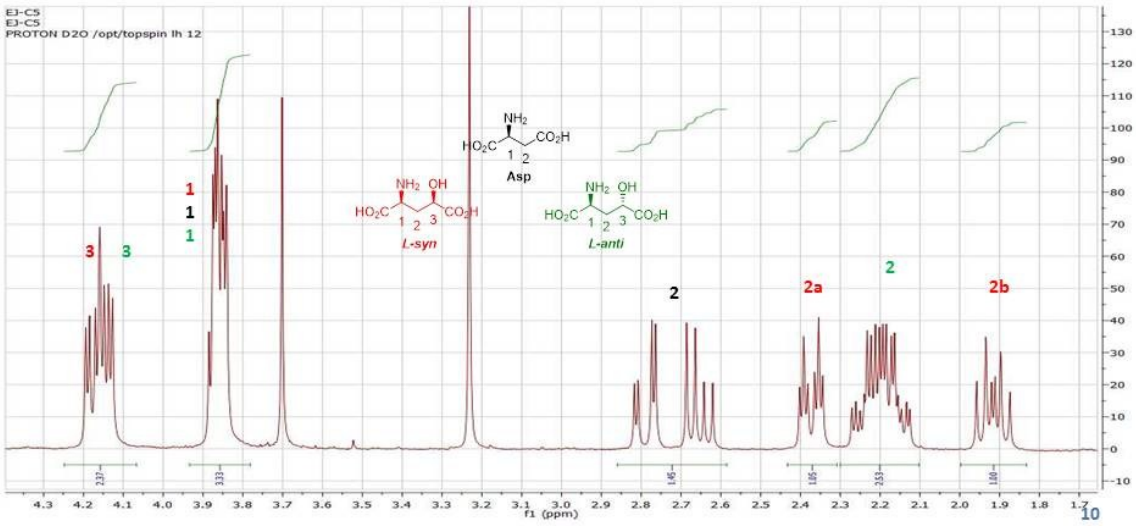


Fig S1 Example of ^1H NMR spectrum obtained with one PyrAL (Uniprot Id. Q2K7V6) after a microscale synthesis experiment.

6. Enzymes production and purification

His-tagged aldolases and transaminases were cloned into plasmid pET22b(+), expressed in *E. coli BL21*Star (DE3) strains and cell lysates prepared as previously described.[3,4] After centrifugation supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to check for recombinant protein production. For IMAC purification, cell lysates were prepared from 1 g of wet cells in 50 mM potassium phosphate (KP), 0.3 M KCl, 10 mM imidazole, pH 8 (10 mL) and poured onto Ni-NTA agarose (10 mL) equilibrated with the same buffer. The column was washed with 50 mM KP, 0.3 M KCl, 20 mM imidazole (100 mL) before elution of the tagged enzyme with 50 mM KP, 0.3 M KCl, 250 mM imidazole (100 mL). Aldolase containing fractions were pooled, dialysed with H_2O (4 L) and lyophilized to give a white powder. Transaminase solutions were dialysed with 50 mM KP, 3M $(NH_4)_2SO_4$, 0.1 mM pyridoxal phosphate (PLP), pH 7,5 (3 x 0.5 L, 3 x 6 h). The final enzyme suspensions were stored at 4°C. Protein concentrations were determined by the Bradford method with bovine serum albumin as the standard (Bio-Rad). Before use, transaminases suspensions were centrifuged and the supernatant discarded. Enzyme activities were determined using the assays described in section 4. Enzymes were stored at 4 °C for several months without noticeable loss of activity. Table S1 summarizes purification results for the various enzymes.

Table S1 Production and activity of the various enzymes (from 1 L cell culture).

Enzyme Family	Enzyme name / Uniprot Id. / MW (g.mol^{-1})	Purified enzyme (mg)	Assay substrates[a]	Total activity (U)	Specific activity (U.mg^{-1})
L-α-TA	AspTA / P00509 44,396	228	HKG + L-Asp	18700	105
L-α-TA	L-TA1 / G0VQA2 45,515	118	HKG + L-Ala	760	3.5
			DHOP + L-Ala	195	0.9
D-α-TA	D-TA2 / E6LHY8 33,251	69	HKG + D-Ala	1050	28
			DHOP + D-Ala	2138	57
PyrAL	AL1 / A0A081HJP9 29,026	51	PA + GA	255	5
PyrAL	AL2 / B7NJZ1 28,222	142	PA + GA	11800	83

[a] HKG = 4-hydroxy-2-oxoglutaric acid; Asp = Aspartic acid; Ala = Alanine; PA = pyruvic acid; GA = glyoxylic acid

7. Kinetic parameters for selected enzymes

Kinetic parameters for TA-catalysed reactions were determined with the assays described in sections 4.1 and 4.2 by varying HKG concentration (0.1 – 10 mM) and with a fixed concentration of amino-donor substrate (20 mM Asp, L-Ala or D-Ala). For PyrAL, 2 series of experiments were performed using the assay described in section 4.3: In the first series variable PA concentrations (1 - 50 mM) were used with 10 mM GA and in the second series, variable GA concentrations (1 – 50 mM) were used with 10 mM PA. Kinetic parameters values and standard errors were calculated from the Hanes-Woolf plot according to the least-squares method and Gauss's error propagation law. Results are summarised in table S2.

Table S2 Kinetic parameters determined for the various enzymes

Enzyme Family	Enzyme name or Uniprot Id.	Substrates	Km (mM)	kcat (s^{-1})	kcat/Km ($s^{-1}.mM^{-1}$)
L-α-TA	AspTA	HKG	0.64 ± 0.09	78 ± 2	123 ± 18
		L-Asp	nd	nd	Nd
L-α-TA	L-TA1 / G0VQA2	HKG	0.04 ± 0.02	2.6 ± 0.1	66 ± 30
		DHOP	0.87 ± 0.15	0.71 ± 0.04	0.81± 0.14
		L-Ala	nd	nd	nd
D-α-TA	D-TA2 / E6LHY8	HKG	3.2 ± 0.4	16 ± 2	5 ± 1
		DHOP	7.8 ± 0.9	32 ± 3	4.1 ± 0.6
		D-Ala	nd	nd	nd
PyrAL	AL1	PA	18 ± 2	3.6 ± 0.3	0.20 ± 0.03

	/ A0A081HJP9	GA	12.7 ± 1.5	2.5 ± 0.2	0.20 ± 0.03
PyrAL	AL2/ B7NJZ1	PA	3.9 ± 0.5	42 ± 2	11 ± 2
		GA	1.8 ± 0.2	39 ± 2	22 ± 3

As shown in Fig S2 and S3, inhibition was observed for both PyrAL with the highest assayed PA and GA concentrations. Consequently, rates measured above 10 mM substrate were not taken into account for kinetic parameters calculations.

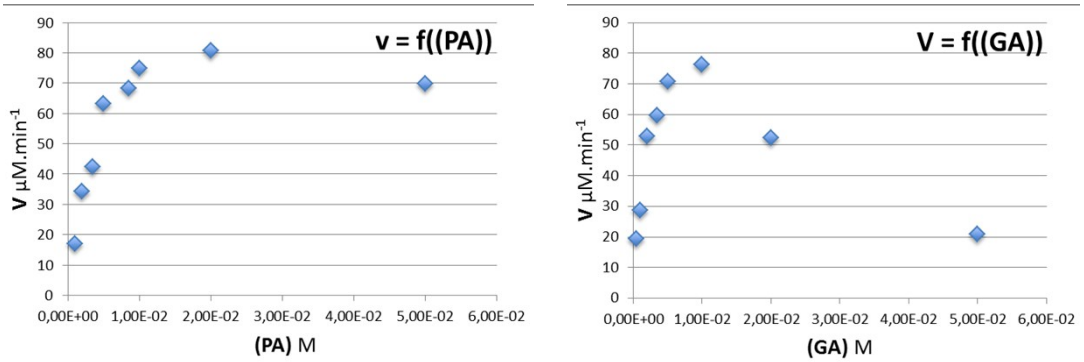


Fig S2 Initial reaction rates measured for AL2 (Uniprot Id. B7NJZ1) at various substrate concentrations.

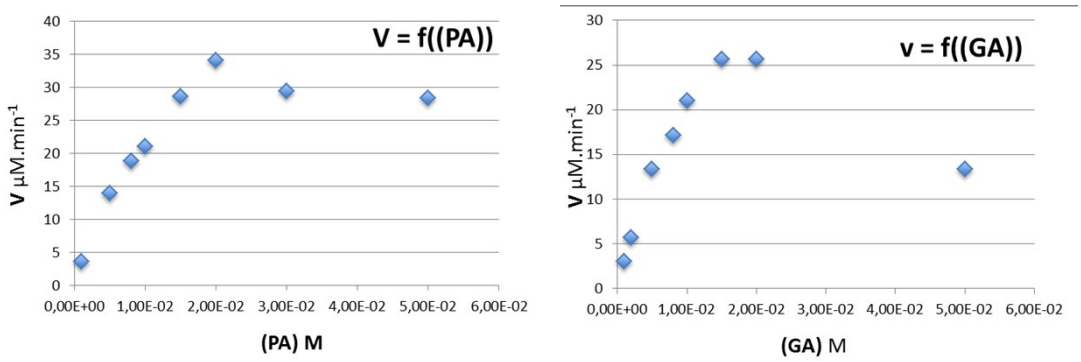


Fig S3 Initial reaction rates measured for AL1 (uniprot Id. A0A081HJP9) at various substrate concentrations.

8. Titration of substrates and products

Pyruvic acid (PA) was titrated in 1 mL total volume containing NADH (0.4 mM), LDH (1U) and PA (≤ 0.3 mM). Initial absorbance (Ai) and final absorbance (Af) were measured before and after addition of PA (10 µL) respectively. PA concentration in the assay was determined using the following equation: (PA) = (Af-Ai/1.01)/6220

4-hydroxy-2-oxoglutaric acid (HKG) was titrated in 1 mL total volume containing NADH (0.4 mM), MDH (1U), HKG (≤ 0.3 mM), Asp 40 mM and AspTA (0.5 U). Initial absorbance (Ai) and final absorbance (Af) were measured before and after addition of AspTA (10 µL) respectively. HKG concentration in the assay was determined using the following equation: (HKG) = (Af-Ai/1.01)/6220

9. Aldolase-Transaminase cascades

9.1 Synthesis of L-*syn*-HGlu: Recycling cascade combining AL1 (Uniprot Id. A0A081HJP9) and AspTA with L-Asp as amino donor substrate.

To a mixture containing 1 M PA (100 µL, 0.1 mmol), 1 M L-Asp (1 mL, 1 mmol), 0.1 M $MnCl_2$ (100 µL, 0.01 mmol), 10 mM PLP (100 µL, 0.01 mmol), 0.1 M KP (2 mL) and H_2O (5.6 mL) was added AL1 (10 mg) and AspTA (0.5 mg). To the gently stirred mixture was added a solution of 1 M GA (1 mL, 1 mmol) over 400 min. using a syringe pump. The reaction mixture was stirred for additional 16 h at room temperature. It was then poured on a column of Dowex®50WX8 (H^+ form, 3 mL). The column was washed with H_2O (20 mL) and the eluted with 1 M NH_3 (20 mL). The ninhydrin positive fractions were pooled and concentrated under reduced pressure. ^{1}H NMR indicated a conversion and d.e. over 95%. The residue was then dissolved in H_2O (4 mL) and poured on a column of Dowex®1X8 (AcO^- form, 10 mL). The column was washed with H_2O (10 mL) and the eluted with a 0.3 - 0.6 M AcOH gradient (100 mL). Fractions containing HGlu were pooled and concentrated under reduced pressure to give a white solid. It was dissolved in H_2O (2 mL) and concentrated again. This operation was repeated to remove AcOH. L-*syn*-HGlu was thus isolated as a white solid (150 mg, 92%). Configuration was assigned by comparison with NMR spectra and optical rotations described in literature for stereoisomers of HGlu.[5–9]

^{1}H NMR (400 MHz, D_2O) δ 4.35 (1H, dd, *J* = 4.0 and 10.0 Hz, H^4), 3.88 (1H, dd, *J* = 5.0 and 8.0 Hz, H^2), 2.39 (1H, ddd, *J* = 4.0, 5.0 and 15.0 Hz, H^{3a}), 1.95 (1H, ddd, *J* = 8.0, 10.0 and 15.0 Hz, H^{3b}); ^{13}C NMR (100 MHz, D_2O) δ 177.3, 173.2 (C^1 and C^5), 68.9 (C^4), 52.8 (C^2), 33.8 (C^3); HRMS (ES-) *m/z* 162.0396 ([M-H]$^-$, calc. for $C_5H_8NO_5$: 162.0397); $[\alpha]_D^{25}$ = + 18 (c 2, H_2O) litt[6] $[\alpha]_D^{25}$ = + 20 (c 2, H_2O).

9.2 Synthesis of L-*anti*-HGlu: Recycling cascade combining AL2 (Uniprot Id. B7NJZ1) and AspTA with L-Asp as amino donor substrate.

The procedure described in section 9.1 was used with AL2 (10 mg) and AspTA (1 mg). GA solution was added over 4 h and the reaction stopped after 8 h. ^{1}H NMR indicated quantitative conversion and d.e. over 98%. L-*anti*-HGlu was thus isolated as a white solid (155 mg, 95%). Configuration was assigned by comparison with NMR spectra and optical rotations described in literature for stereoisomers of HGlu.[5–9]

^{1}H NMR (400 MHz, D_2O) δ 4.18 (1H, dd, *J* = 4.0 and 8.5 Hz, H^4), 3.89 (1H, dd, *J* = 3.5 and 8.5 Hz, H^2), 2.30-2.16 (2H, m, H^3); ^{13}C NMR (100 MHz, D_2O + LiOH, PH 10) δ 179.9, 174.1 (C^1 and C^5), 70.0 (C^4), 53.1 (C^2), 34.1 (C^3); HRMS (ES-) *m/z* 162.0395 ([M-H]$^-$, calc. for $C_5H_8NO_5$: 162.0397); $[\alpha]_D^{25}$ = -12 (c 1, H_2O), litt[6] $[\alpha]_D^{25}$ = -12.5 (c 2, H_2O).

9.3 Synthesis of DHNV: Recycling cascades combining AL1 (Uniprot Id. A0A081HJP9), AL2 (Uniprot Id. B7NJZ1), L-TA1 (Uniprot Id. G0VQA2) and D-TA2 (Uniprot Id. E6LHY8).

To a mixture containing 0.1 M PA (130 µL, 0.013 mmol), 0.5 M GL (260 µL, 0.13 mmol), 0.5 M L- or D-Ala (260 µL, 0.13 mmol), 0.1 M $MgCl_2$ (26 µL, 2.6 µmol), 10 mM PLP (26 µL, 0.26 µmol), and H_2O (1.7 mL) adjusted to pH 7.8 with 0.1 M NaOH, was added 0.33 mg.mL^{-1} AL1 or 12 mg.ml^{-1} AL2 (200 µL) and L-TA1 (4 mg) or D-TA2 (0.33 mg). The reaction mixture was stirred for 4 h at 20 °C. The mixture was poured on a column of Dowex®50WX8 (H^+ form, 1 mL). The column was washed with H_2O (5 mL) and then eluted with 1 M NH_3 (10 mL). The ninhydrin positive fractions were pooled and concentrated under reduced pressure. The residue was analysed by ^{1}H NMR to determine conversion and stereoselectivity. Products configuration was assigned by comparison with spectroscopic data published for L-*syn* or *anti*-DHNV.[10–12]

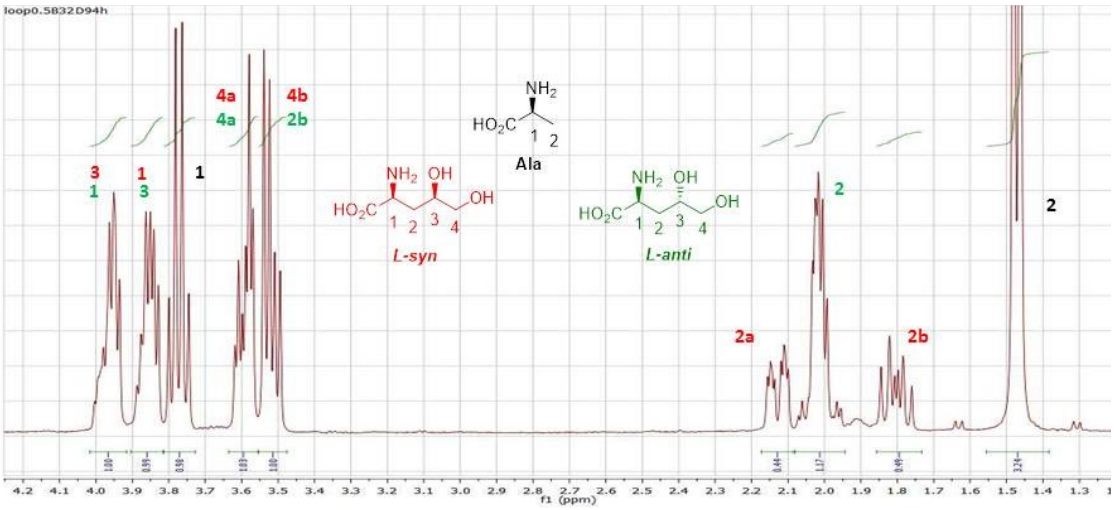


Fig S4 Example of ^{1}H NMR spectrum obtained with AL2 and L-TA1

9.4 Synthesis of D-*anti*-DHNV: Recycling cascades combining AL1 (Uniprot Id. A0A081HJP9) and D-TA2 (Uniprot Id. E6LHY8) in various conditions.

To a mixture containing 1 M PA (10, 0.01 mmol), 0.5 M GL (200-320 µL, 0.1-0.16 mmol), 1 M L- or D-Ala (0.1 mL, 0.1 mmol), 0.1 M $MgCl_2$ (10 µL, 1 µmol), 10 mM PLP (10 µL, 0.1 µmol), and H_2O (0.43-0.62 mL, 1 mL final volume) adjusted to pH 7.8 with 0.1 M NaOH, was added 2 mg.mL^{-1} AL1 (12.5 - 200 µL, 0.025 - 0.4 mg) and D-TA2 (0.33, 0.65 or 1 mg). The reaction mixture was stirred for 24 h at 20 °C. pH was controlled and adjusted to 7.8 at 30 min, 1, 2, 3 and 4 h. At 4 h and 24 h, 0.5 mL of the mixture was poured on a column of Dowex®50WX8 (H^+ form, 1 mL). The column was washed with H_2O (5 mL) and then eluted with 1 M NH_3 (10 mL). The ninhydrin positive fractions were pooled and concentrated under reduced pressure. The residue was analysed by ^{1}H NMR to determine conversion and stereoselectivity. Products configuration was assigned by comparison with spectroscopic data published for L-*syn* or *anti*-DHNV.[10–12]

9.5 Synthesis of D-*anti*-DHNV: Recycling cascade combining AL1 (Uniprot Id. A0A081HJP9) and D-TA2 (Uniprot Id. E6LHY8) on 1 mmol scale.

A mixture containing 1 M PA (100 µL, 0.1 mmol), 1 M D-Ala (1 mL, 1 mmol), 0.1 M $MgCl_2$ (100 µL, 10 µmol), 10 mM PLP (100 µL, 1 µmol), and H_2O (6.9 mL) was adjusted to pH 7.8 with 0.5 M NaOH. To this solution was added 1 mg.mL^{-1} AL1 (0.2 mL, 0.2 mg) and D-TA2 (6.5 mg). To the gently stirred mixture was added a solution of 1 M GL (1.6 mL, 1.6 mmol) over 4 h using a syringe pump. The reaction mixture was stirred for additional 5 h at room temperature. It was then poured on a column of Dowex®50WX8 (H^+ form, 3 mL). The column was washed with H_2O (20 mL) and the eluted with 1 M NH_3 (20 mL). The ninhydrin positive fractions were pooled and concentrated under reduced pressure. ^{1}H NMR indicated a conversion of 81% and a 5:95 *syn*/*anti* ratio. The residue was dissolved in 5 mL concentrated HCl and heated at 100 °C for 3 h before concentration under reduced pressure.^{1}H NMR analysis indicated a complete conversion of DHNV into the corresponding 5-membered ring lactone:[13] ^{1}H NMR (400 MHz, D_2O) δ 4.75-4.85 (1H, m, H^4), 4.51 (1H, dd, *J* = 9.0 and 12.0 Hz, H^2), 3.94 (1H, dd, *J* = 2.5 and 13.0 Hz, H^{5a}), 3.67 (1H, dd, *J* = 5.0 and 13.0 Hz, H^{5b}), 2.77 (1H, ddd, *J* = 6.0, 9.0 and 13.0 Hz, H^{3a}), 2.21 (1H, ddd, *J* = 10.0, 12.0 and 13.0 Hz, H^{3b}); ^{13}C NMR (100 MHz) δ 173.0 (C^1), 79.8 (C^4), 61.3 (C^5), 49.4 (C^2), 28.1 (C^3). The residue was dissolved in H_2O (4 mL) and the

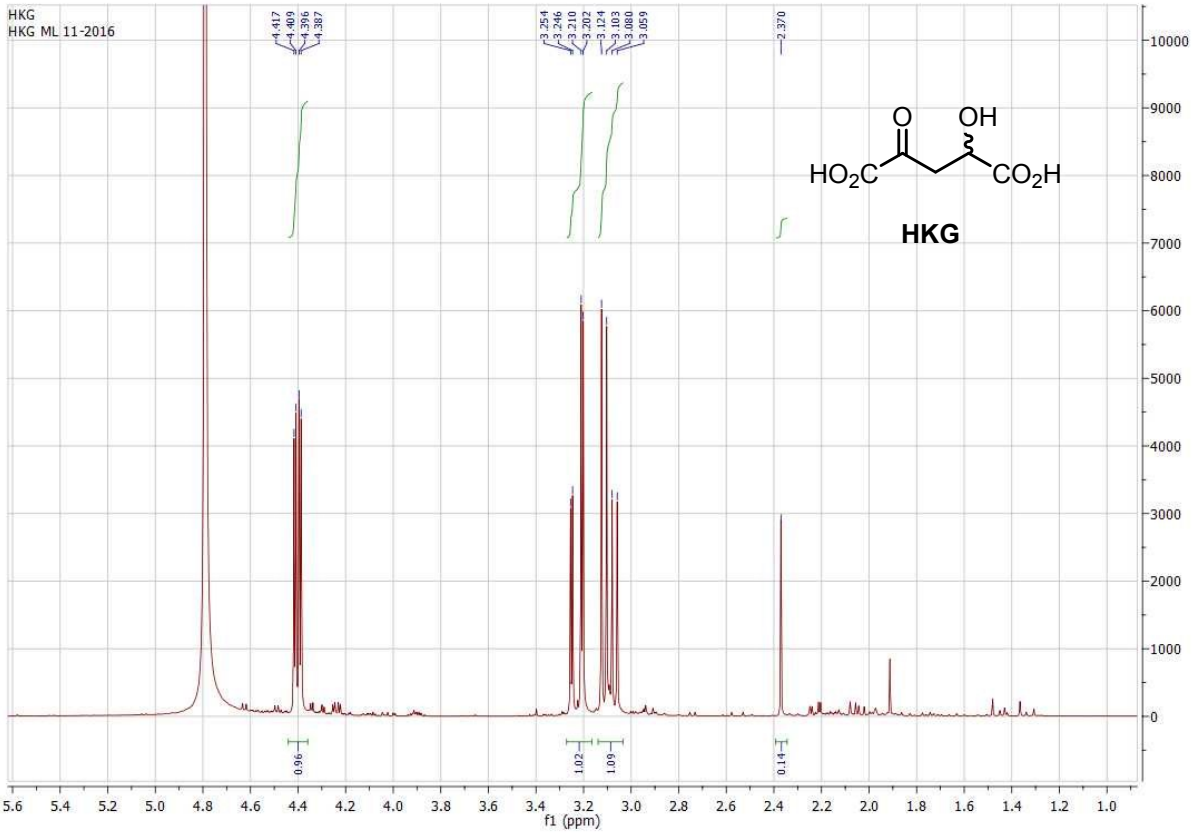
solution adjusted to pH 4 with 0.1 M NaOH before adsorption on a column of Dowex®50WX8 (Na^+ form, 3 mL). D-Ala was selectively eluted with H_2O (10 mL) before elution with 0.1 M NaOH which allowed lactone saponification and DHNV elution. The ninhydrin positive fractions containing DHNV were pooled and adusted to pH 7 with 1 M HCl. The solution was then poured on a column of Dowex®50WX8 (H^+ form, 3 mL). The column was washed with H_2O (10 mL) and then eluted with 1 M NH_3. The ninhydrin positive fractions containing DHNV were pooled and concentrated under reduced pressure to give D-*anti*-DHNV (124 mg, 83%, 7:93 *syn*/*anti* ratio). Product configuration was assigned by comparison with spectroscopic data published for L-*syn* or *anti*-DHNV.[10–12]

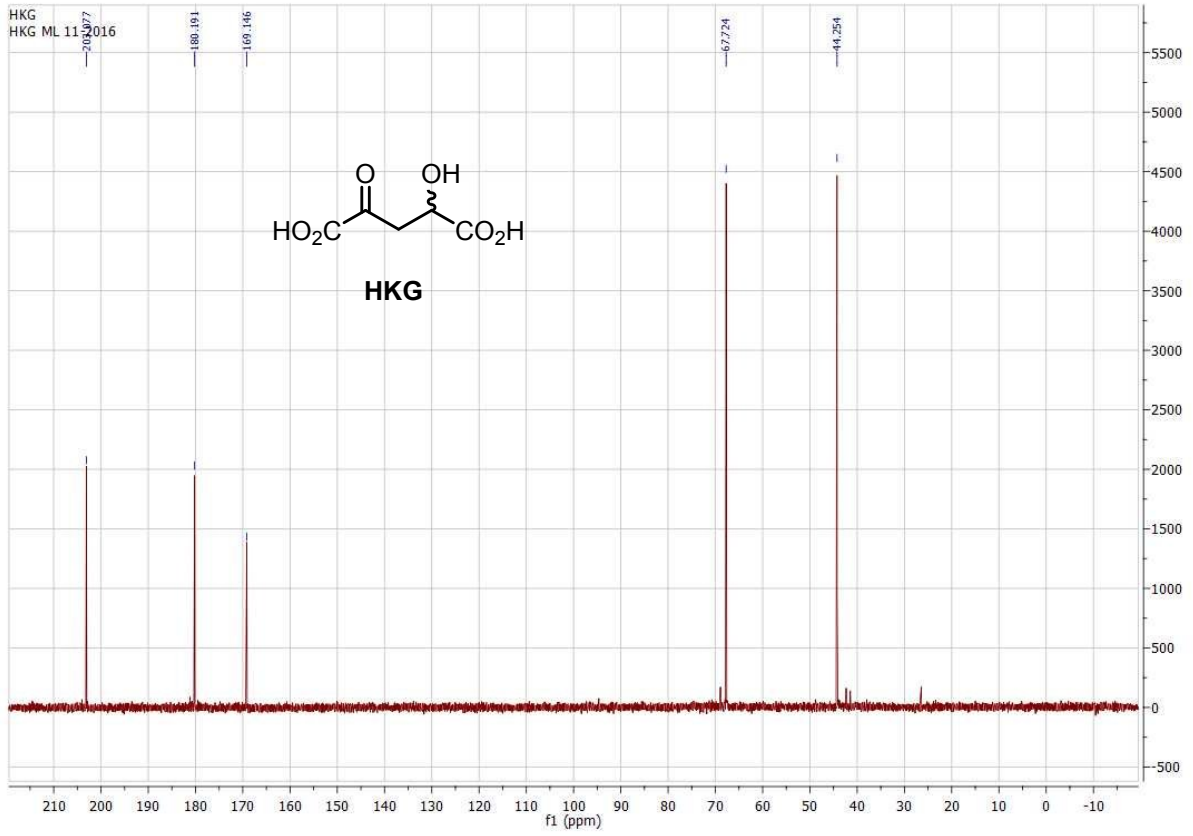
^{1}H NMR (400 MHz, D_2O + LiOH, pH 10) δ 3.91 (1H, dd, *J* = 5.0 and 6.5 Hz, H^2), 3.83 (1H, m, H^4), 3.56 (1H, dd, *J* = 4.0 and 12.0 Hz, H^{5a}), 3.48 (1H, dd, *J* = 6.0 and 12.0 Hz, H^{5b}), 2.03-1.92 (2H, m, H^3); ^{13}C NMR (100 MHz, D_2O + LiOH, PH 10) δ 174.2 (C^1), 68.9 (C^4), 65.2 (C^5), 52.7 (C^2), 32.3 (C^3); HRMS (ES-) *m/z* 148.0600 ($[M-H]^-$, calc. for $C_5H_{10}NO_4$:148.0604); $[\alpha]_D^{20}$ = + 19 (c 1.9, H_2O) litt[6] for L-*anti*-DHNV: $[\alpha]_D^{23}$ = - 19.5 (c 1.7, H_2O)

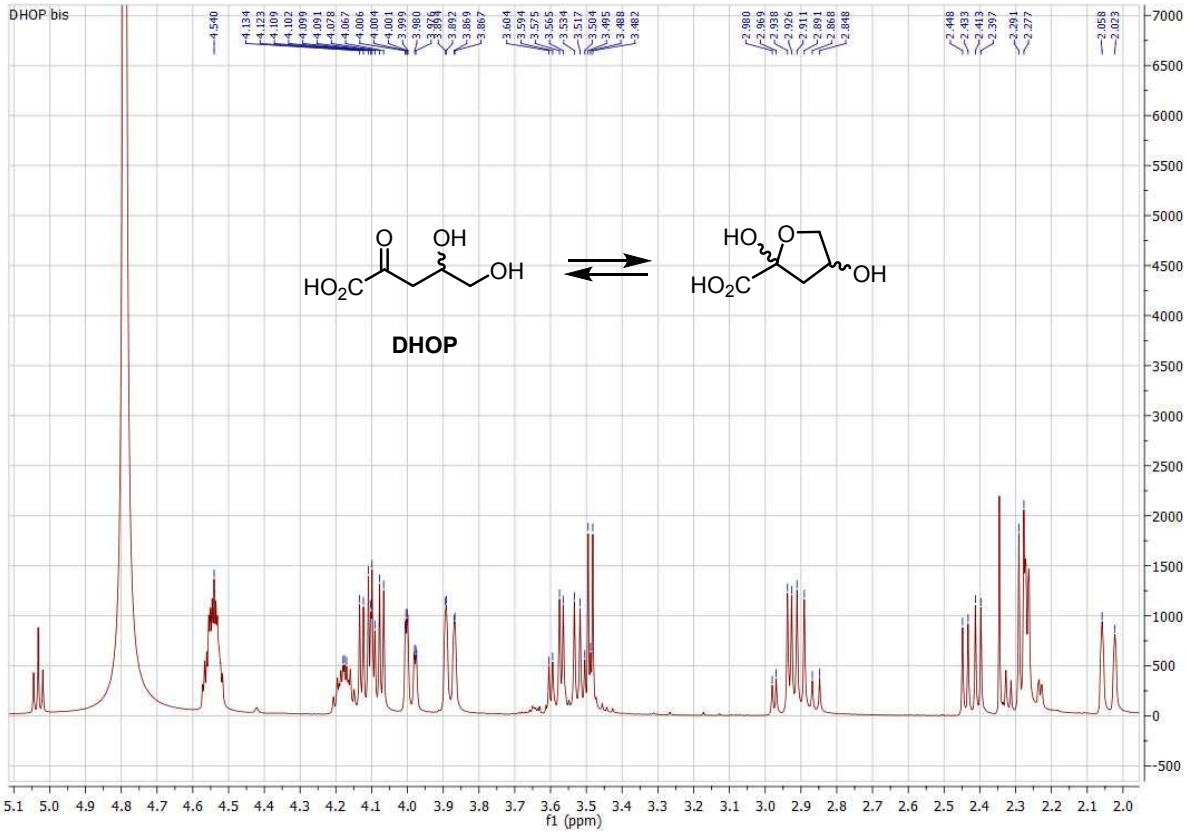
9.6 Two-step cascade combining AL1 (Uniprot Id. A0A081HJP9) and D-TA2 (Uniprot Id. E6LHY8).

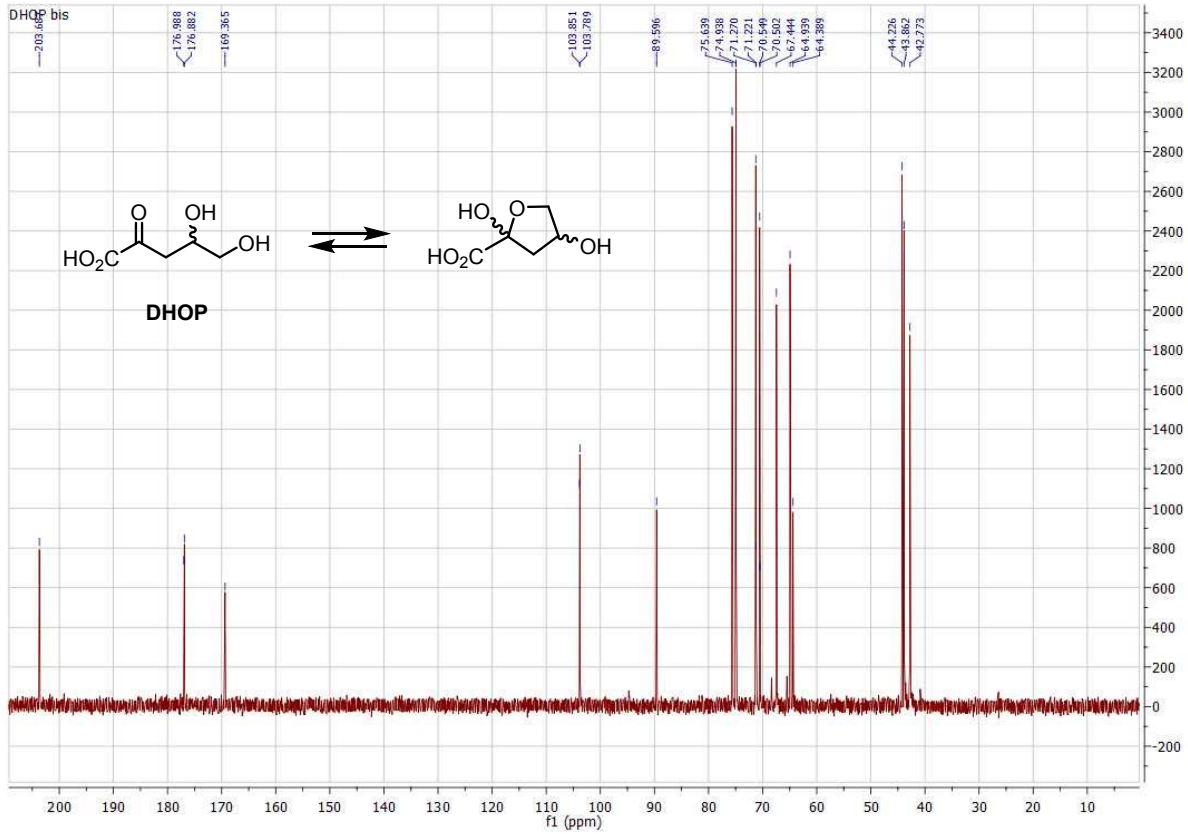
A mixture containing 1 M PA (0.1 mL, 0.1 mmol), 1 M GA (0.12 mL, 0.12 mmol), 1 M D-Ala (0.1 mL, 0.1 mmol), 0.1 M $MgCl_2$ (0.1 mL, 10 µmol), 10 mM PLP (0.1 mL 1 µmol) and H_2O (0.38 mL) was adjusted to pH 7.8 before addition of a solution of 1 mg.mL^{-1} AL1 (0.1 mL, 0.1 mg). The solution was stirred at 20 °and pH was controlled and adjusted to 7.8 with 0.1 M NaOH every hour. After 4 h, D-TA2 (0.65 mg) was added to the reaction mixture. After 8 h, the reaction mixture was poured on a column of Dowex®50WX8 (H^+ form, 1 mL). The column was washed with H_2O (5 mL) and then eluted with 1 M NH_3 (10 mL). The ninhydrin positive fractions were pooled and concentrated under reduced pressure. The residue was analysed by ^{1}H NMR to determine conversion and stereoselectivity. Products configuration was assigned by comparison with spectroscopic data published for L-*syn* or *anti*-DHNV.[10–12]

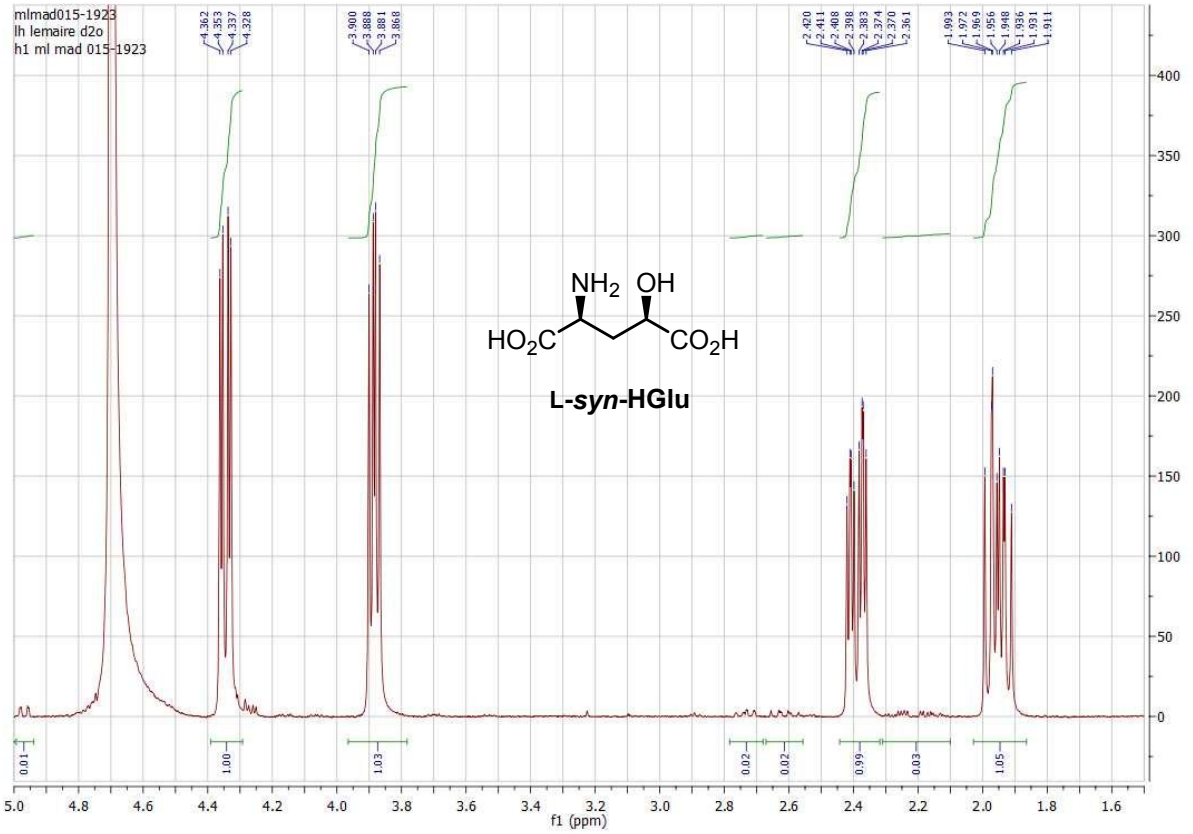
9.7 NMR spectra of products

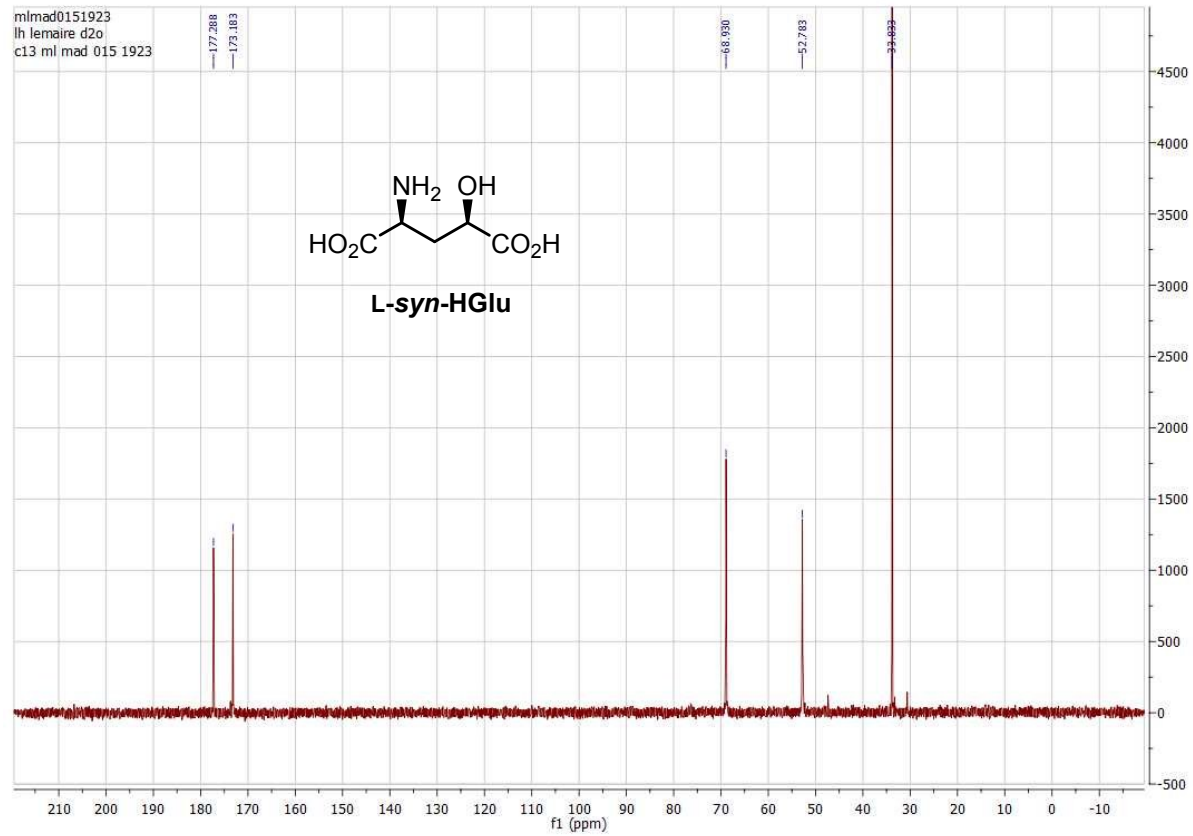


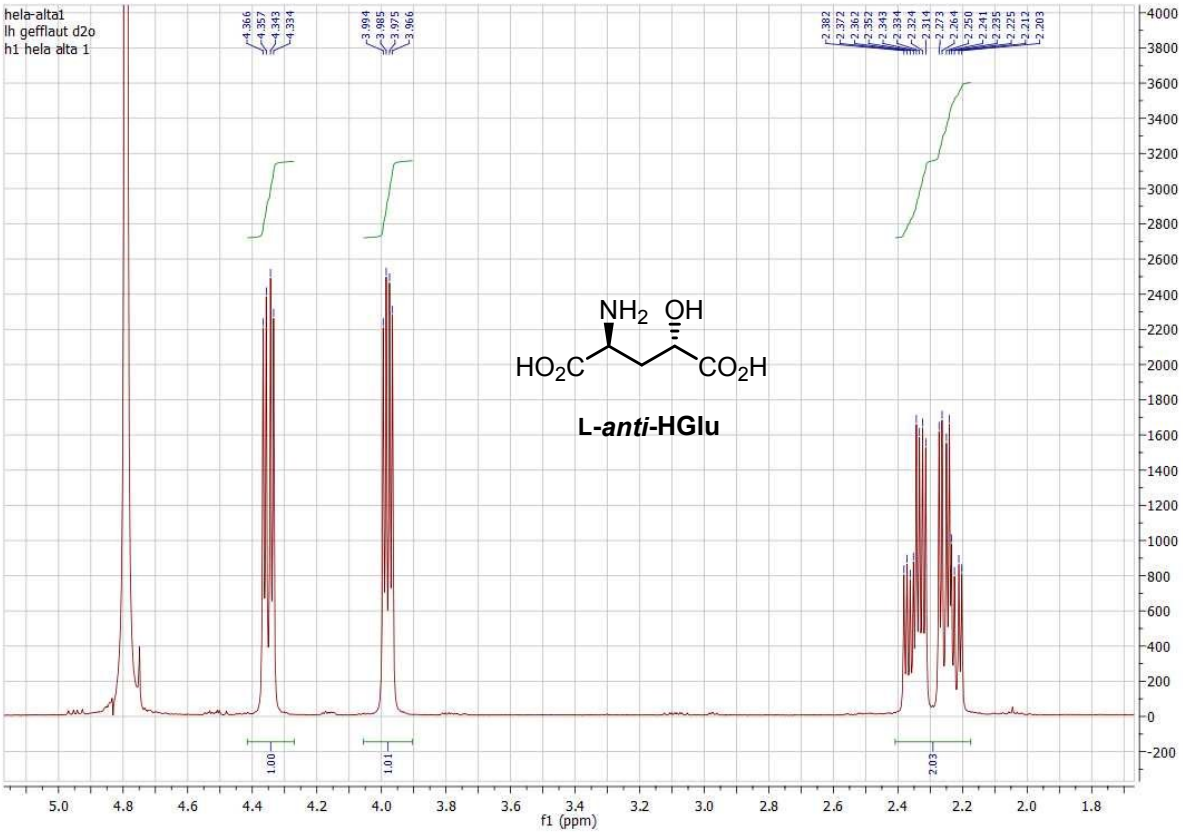



DHOP bis
DHOP
f1 (ppm)

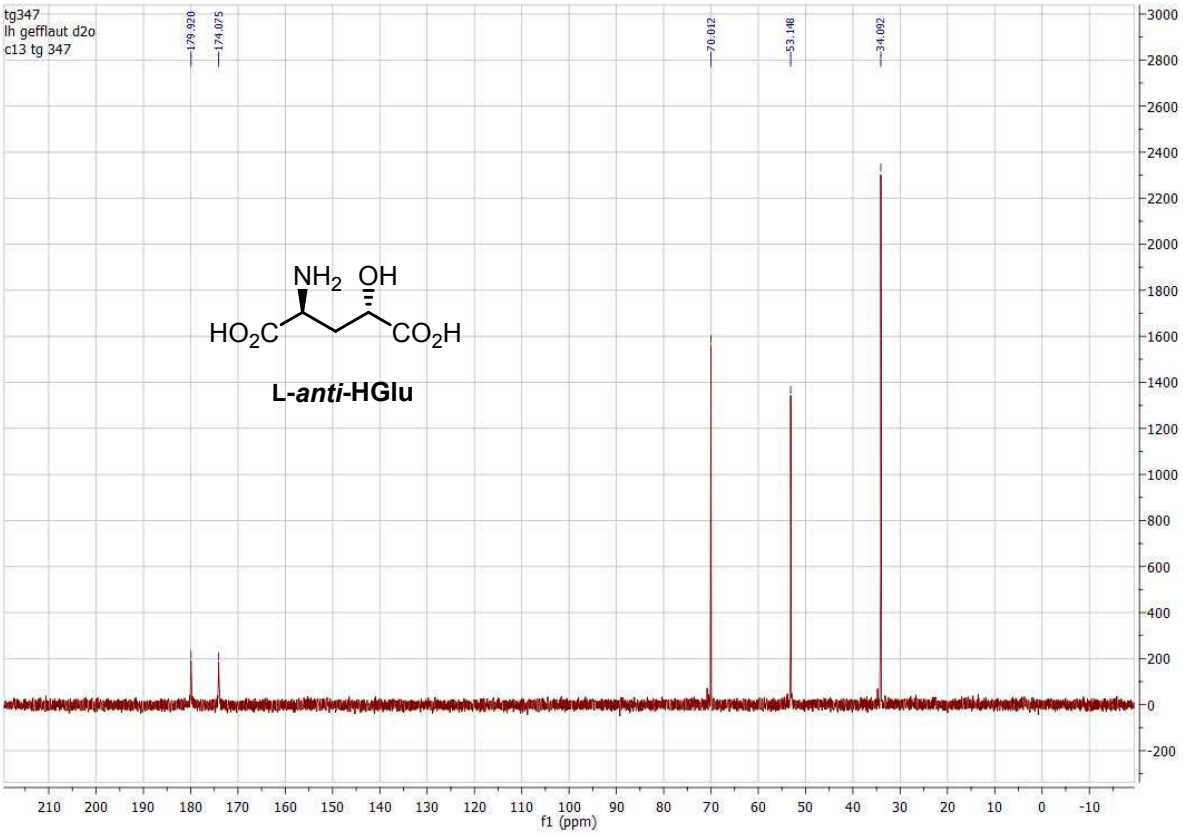

DHOP bis
DHOP
f1 (ppm)


mlmad015-1923
lh lemaire d2o
h1 ml mad 015-1923
NH2 OH
HO2C CO2H
L-syn-HGlu
f1 (ppm)

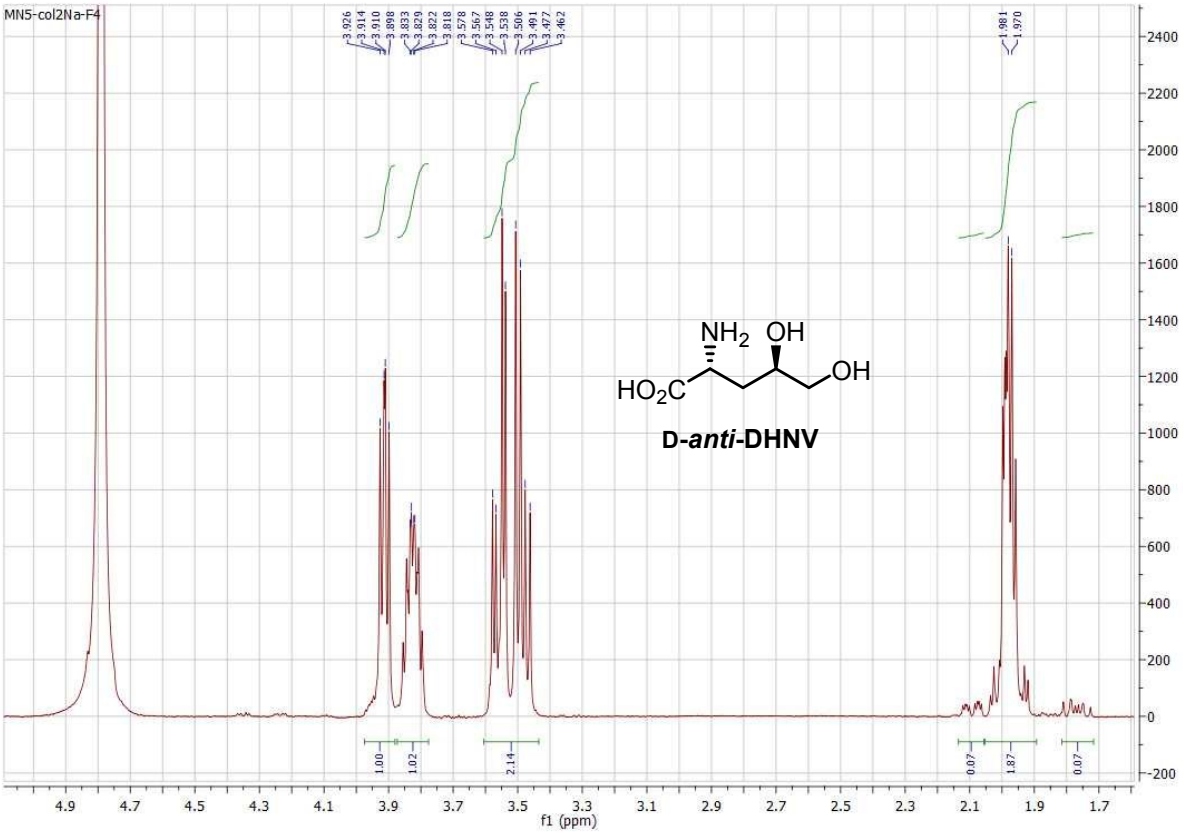

mlmad0151923
lh lemaire d2o
c13 ml mad 015 1923
NH2 OH
HO2C CO2H
L-syn-HGlu
f1 (ppm)

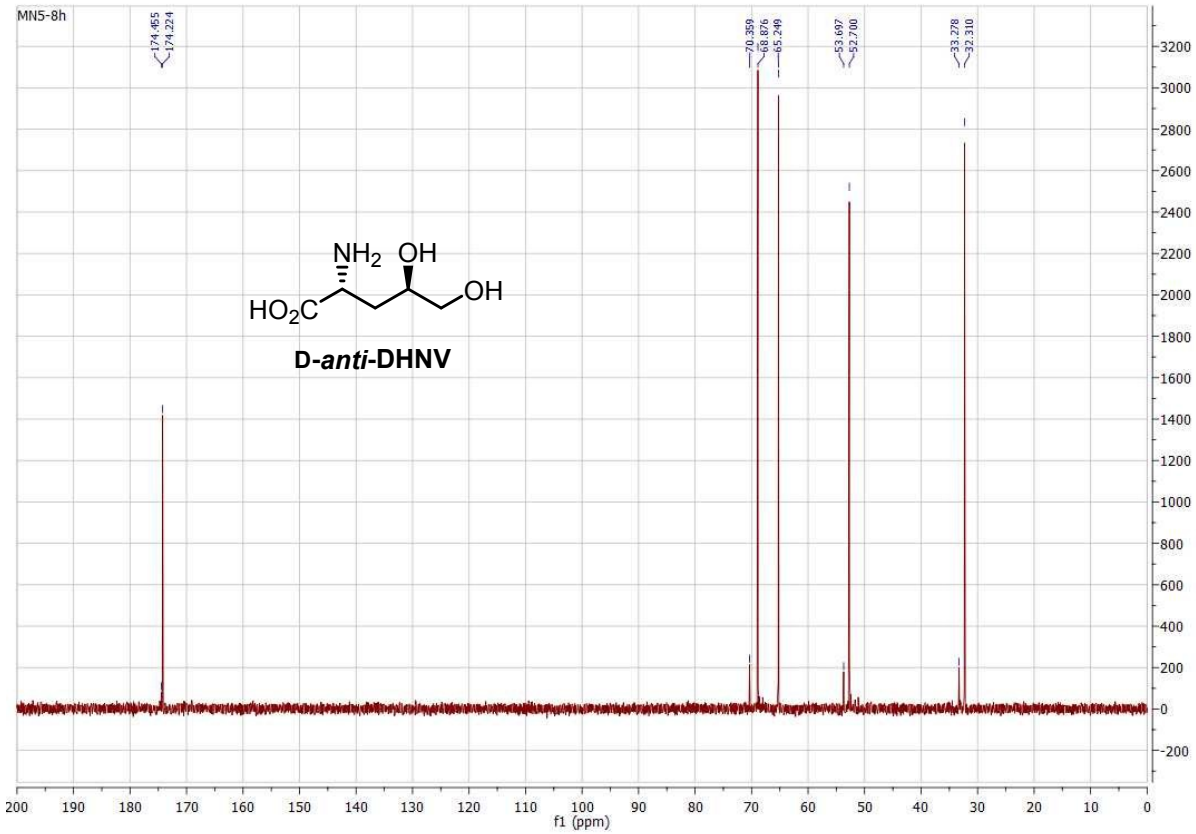


L-*anti*-HGlu



L-*anti*-HGlu


MN5-col2Na-F4
3.926
3.914
3.910
3.898
3.833
3.829
3.822
3.818
3.578
3.567
3.548
3.538
3.506
3.491
3.477
3.462
1.981
1.970
NH2 OH
HO2C
OH
D-anti-DHNV
1.00
1.02
2.14
0.07
1.87
0.07
f1 (ppm)


MN5-8h
174.465
174.224
70.359
68.876
65.249
53.697
52.700
33.278
32.310
NH2 OH
HO2C
OH
D-anti-DHNV
f1 (ppm)

References

1 A. Ruffo, E. Testa, Adinolfia and G. Pelizza, *Biochem. J.*, 1962, **85**, 588–593.
2 A. C. Stoolmiller and R. H. Abeles, *J. Biol. Chem.*, 1966, **241**, 5764–5771.
3 C. Vergne-Vaxelaire, F. Bordier, A. Fossey, M. Besnard-Gonnet, A. Debard, A. Mariage, V. Pellouin, A. Perret, J.-L. Petit, M. Stam, M. Salanoubat, J. Weissenbach, V. De Berardinis and A. Zaparucha, *Adv. Synth. Catal.*, 2013, **355**, 1763–1779.
4 V. de Berardinis, C. Guérard-Hélaine, E. Darii, K. Bastard, V. Hélaine, M. Stam, A. Mariage, J.-L. Petit, N. Poupard, I. Sanchez-Moreno, T. Gefflaut, M. Salanoubat and M. Lemaire, *Green Chem.*, 2017.
5 P. Lorenz, F. R. Stermitz and L. D. Ismail, *Phytochemistry*, 1999, **52**, 63–66.
6 Y. K. Lee and T. Kaneko, *Bull. Chem. Soc. Jpn.*, 1973, **46**, 3494–3498.
7 E. P. Kristensen, L. M. Larsen, O. Olsen, H. Sørensen and C. R. Enzell, *Acta Chem. Scand.*, 1980, **34b**, 497–504.
8 V. P. Krasnov, L. V. Alekseeva, N. A. Firsova, I. K. Kodess and N. L. Burde, *Pharm. Chem. J.*, 1984, **18**, 369–372.
9 C. Ducrocq, P. D.-L. Maréchal and R. Azerad, *J. Label. Compd. Radiopharm.*, 1985, **22**, 61–70.
10 R. G. Soengas, J. C. Estévez and R. J. Estévez, *Tetrahedron Asymmetry*, 2003, **14**, 3955–3963.
11 J. Ariza, M. Díaz, J. Font and R. M. Ortuño, *Tetrahedron*, 1993, **49**, 1315–1326.
12 K. Isono, K. Asahi and S. Suzuki, *J. Am. Chem. Soc.*, 1969, **91**, 7490–7505.
13 J. Ariza, M. Díaz, J. Font and R. M. Ortuño, *Tetrahedron*, 1993, **49**, 1315–1326.