

# **Synthesis and Structure Reassignment of Malylglutamate, a Recently Discovered Earthworm Metabolite**

Corey M. Griffith,<sup>†§</sup> Abigail Feciu,<sup>‡§</sup> Cynthia K. Larive,<sup>‡</sup> David B. C. Martin<sup>\*‡</sup>

<sup>†</sup>Environmental Toxicology Graduate Program and <sup>‡</sup>Department of Chemistry

University of California, Riverside, California 92521, United States

## Table of Contents

<b>Materials and Methods .....</b>	<b>S3</b>
<b>Synthesis and Characterization of Coupling Partners.....</b>	<b>S3</b>
<b>Synthesis and Characterization of Benzylated compounds .....</b>	<b>S3-S5</b>
<b>Synthesis and Characterization of natural product isomers .....</b>	<b>S5-S7</b>
<b>NMR Acquisition and Data Processing for Earthworm Samples .....</b>	<b>S7</b>
<b>References .....</b>	<b>S8</b>
<b><math>^1\text{H}</math> and <math>^{13}\text{C}</math> NMR Spectra .....</b>	<b>S9-S17</b>

## Materials and Methods:

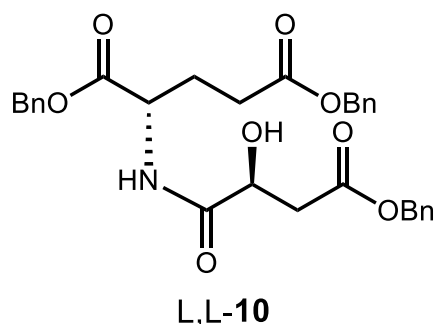
All reactions were carried out under an inert atmosphere of nitrogen in oven dried or flame dried glassware with magnetic stirring, unless otherwise noted. Solvents were dried by passage through columns of activated alumina. All starting materials were prepared according to known literature procedures or used as obtained from commercial sources, unless otherwise indicated. Reactions were monitored by thin-layer chromatography (TLC) and carried out on 0.25 mm coated commercial silica gel plates (Analtech TLC Uniplates, F<sub>254</sub> precoated glass plates with organic fluorescent binder) using UV light as visualizing agent and KMnO<sub>4</sub> and heat as a developing agent. Flash chromatography was performed on silica gel (Silicycle, SiliaFlash P60, 230-400 mesh).

## Synthesis and Characterization of Coupling Partners

**L-6, D-6, L-8, L-9, L-11, and D-11** were synthesized according to known literature procedures.<sup>1-4</sup>

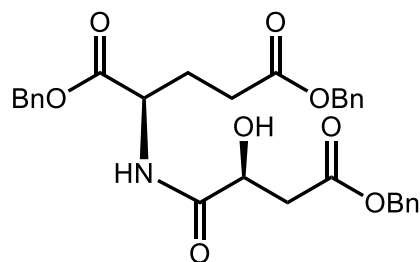
## Synthesis and Characterization of Benzylated Compounds

**General Procedure for Peptide Coupling Reactions (A):** To a flame dried flask equipped with a magnetic stir bar were sequentially added acid (1.0 eq), amine (1.2 eq), HOBT (1.2 eq), EDCI (2.0 eq) and DMF (60 mM). The flask was sealed with a septum and degassed by sparging with N<sub>2</sub>. The reaction mixture was stirred for 10 minutes followed by addition of Et<sub>3</sub>N (4.0 eq) dropwise. The reaction mixture was stirred for 24 hours and diluted with EtOAc. The organic layer was washed with water. The layers were separated and the aqueous layer was extracted with EtOAc (2x). The combined organic layers were washed with brine and dried over Na<sub>2</sub>SO<sub>4</sub>. The solvent was removed in vacuo and the crude residue was purified using silica gel chromatography to provide benzylated products as a white solid.



**(S)-Dibenzyl 2-((S)-4-(benzyloxy)-2-hydroxy-4-oxobutanamido)pentanedioate (L,L-10)**

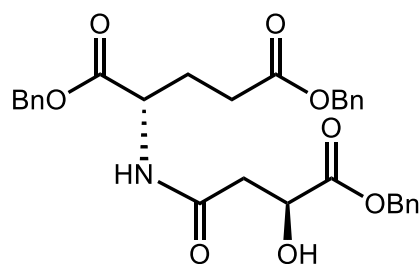
Prepared according to General Procedure A using acid **L-9** (22 mg, 0.10 mmol) and amine **L-6** (60 mg, 0.12 mmol) as the substrates. Reaction time: 24 h. The crude residue was purified by column chromatography on silica gel using EtOAc to afford **L,L-10** as a white solid (22 mg, 42% yield). IR (film) 3388, 3063, 3032, 1735, 1731  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  1.98-2.10 (m, 1H), 2.20-2.34 (m, 1H), 2.35-2.50 (m, 2H), 2.82 (dd,  $J = 7.88, 17.44$  Hz, 1H), 2.94 (dd,  $J = 3.88, 17.48$  Hz, 1H), 3.60-3.66 (m, 1H), 4.40-4.42 (m, 1H), 4.64-4.72 (m, 1H), 5.10 (s, 2H), 5.13 (s, 2H), 5.16 (s, 2H), 7.27-7.39 (m, 15H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  172.6, 172.4, 172.2, 171.4, 135.9, 135.4, 135.3, 133.9, 128.8, 128.7, 128.7, 128.6, 128.5, 128.4, 128.4, 77.5, 77.2, 76.9, 72.2, 68.6, 67.5, 67.5, 67.0, 66.7, 51.6, 38.8, 38.4, 30.3, 29.3, 27.4, 27.3; HRMS (ESI)  $m/z$  calcd. for  $\text{C}_{30}\text{H}_{32}\text{NO}_8$  ( $\text{M}+\text{H}$ ) $^+$  534.2122, found 534.2140.



**L,D-10**

**(*R*)-Dibenzyl 2-((*S*)-4-(benzyloxy)-2-hydroxy-4-oxobutanamido)pentanedioate (**L,D-10**)**

Prepared according to General Procedure A using acid **L-9** (100 mg, 0.446 mmol) and amine **D-6** (267 mg, 0.535 mmol). Reaction time: 24 h. The crude residue was purified by column chromatography on silica gel with EtOAc to afford **L,D-10** as a white solid (63 mg, 26% yield). IR (film) 3389, 3033, 2948, 1731, 1715  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  2.00-2.10 (m, 1H), 2.20-2.33 (m, 1H), 2.35-2.50 (m, 2H), 2.74 (dd,  $J = 8.72, 17.44$  Hz, 1H), 2.94 (dd,  $J = 3.6, 17.56$  Hz, 1H), 3.65 (d,  $J = 4.92$  Hz, 1H), 4.40-4.48 (m, 1H), 4.62-4.72 (m, 1H), 5.09 (s, 2H), 5.15 (s, 2H), 5.16 (s, 2H), 7.28-7.40 (m, 15H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  172.6, 172.0, 171.4, 164.9, 138.6, 135.9, 135.4, 133.9, 128.8, 128.7, 128.7, 128.6, 128.5, 128.5, 128.4, 128.0, 127.8, 77.5, 77.2, 76.9, 72.3, 68.6, 67.6, 67.3, 67.1, 66.7, 51.6, 38.3, 29.9, 27.4; HRMS (ESI)  $m/z$  calcd. for  $\text{C}_{30}\text{H}_{32}\text{NO}_8$  ( $\text{M}+\text{H}$ ) $^+$  534.2122, found 534.2137.

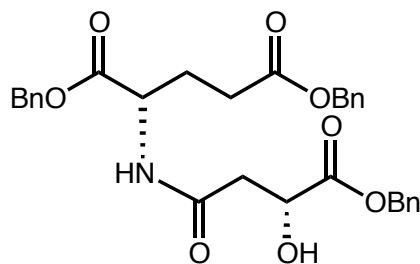


**L,L-S1**

**(*S*)-Dibenzyl 2-((*S*)-4-(benzyloxy)-3-hydroxy-4-oxobutanamido)pentanedioate**

Prepared according to General Procedure A using acid **L-11** (500 mg, 2.23 mmol) and amine **L-6** (1.34 g, 2.67 mmol). Reaction time: 24 h. The crude residue was purified by column chromatography on silica gel using EtOAc to afford **L,L-S1** as a white solid (868 mg, 73% yield). IR (film) 3360, 2915, 1745, 1715, 1170  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  1.94-2.06 (m, 1H), 2.16-2.27 (m, 1H), 2.33-2.48 (m, 1H), 2.62 (dd,  $J = 7.16, 15.32$  Hz, 1H), 2.72 (dd,  $J$

= 3.68, 15.32 Hz, 1H), 3.61 (bs, 1H), 4.50 (dd,  $J = 3.72, 7.12$  Hz, 1H), 4.63-4.70 (m, 1H), 5.09 (s, 2H), 5.15 (s, 2H), 5.20 (s, 2H), 6.56 (d,  $J = 7.84$ , 1H), 7.28-7.39 (m, 15H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  173.3, 172.8, 171.6, 170.0, 135.8, 135.2, 128.8, 128.7, 128.7, 128.6, 128.5, 128.5, 77.5, 77.2, 67.9, 67.8, 67.8, 67.6, 66.7, 51.9, 40.0, 31.0, 30.3, 27.3; HRMS (ESI)  $m/z$  calcd. for  $\text{C}_{30}\text{H}_{32}\text{NO}_8$  ( $\text{M}+\text{H}$ ) $^+$  534.2122, found 534.2119.



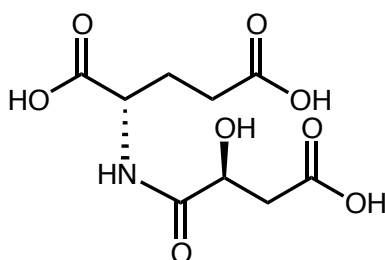
**D,L-S1**

**(S)-Dibenzyl 2-((R)-4-(benzyloxy)-3-hydroxy-4-oxobutanamido)pentanedioate**

Prepared according to General Procedure A using acid **D-11** (400 mg, 1.78 mmol) and amine **L-6** (1.07 g, 2.14 mmol). Reaction time: 24 h. The crude residue was purified by column chromatography on silica gel EtOAc to afford **D,L-S1** as a white solid (683 mg, 72% yield). IR (film) 3352, 3065, 2853, 1733  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  1.94-2.06 (m, 1H), 2.16-2.28 (m, 1H), 2.32-2.48 (m, 2H), 2.63 (dd,  $J = 7.12, 15.28$  Hz, 1H), 2.72 (dd,  $J = 3.72, 15.32$  Hz, 1H), 3.55 (d,  $J = 5.52$  Hz, 1H), 4.47-4.53 (m, 1H), 4.62-4.70 (m, 1H), 5.09 (s, 2H), 5.15 (s, 2H), 5.21 (s, 2H), 6.53 (d,  $J = 7.92$  Hz, 1H), 7.30-7.39 (m, 15H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  177.7, 173.3, 172.3, 170.4, 135.5, 135.1, 128.70, 128.6, 128.5, 128.4, 128.4, 128.3, 77.5, 77.3, 76.9, 67.7, 67.4, 66.8, 66.8, 51.8, 38.8, 30.8, 30.2, 27.2, 23.5; HRMS (ESI)  $m/z$  calcd. for  $\text{C}_{30}\text{H}_{32}\text{NO}_8$  ( $\text{M}+\text{H}$ ) $^+$  534.2122, found 534.2130.

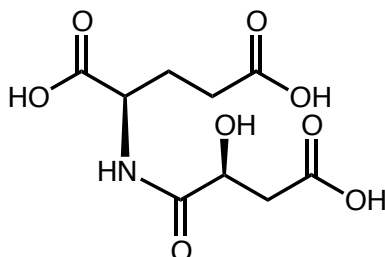
## Synthesis and Characterization of Natural Product Isomers

**General Procedure for Hydrogenolysis (B):** To an oven dried vial equipped with a magnetic stir bar were sequentially added fully benzylated precursor and 10% Pd/C (5 or 10 mol %). The vial was sealed with a septum and pumped and back filled 3 times with nitrogen. This was followed by addition of dry ethanol (0.026 M) and careful addition of  $\text{H}_2$  via balloon. The reaction mixture was stirred for 6 hours and then filtered through celite, washing with DCM. Solvent was removed in vacuo without further purification.



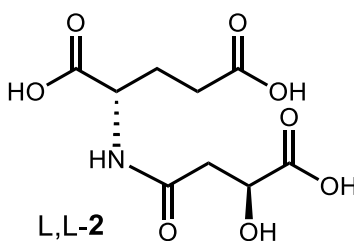
**(S)-2-((S)-3-Carboxy-2-hydroxypropanamido)pentanedioic acid (L,L-1)**

Prepared according to General Procedure B using **L,L-10** (126 mg, 0.236 mmol) to afford **L,L-1** as a white solid (46.0 mg, 73% yield). IR (film) 3396, 2844, 1710, 1450, 1361  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$  4.58 (dd,  $J = 4.0, 8.0$  Hz, 1H), 4.47 (dd,  $J = 4.0, 8.0$  Hz, 1H), 2.90 (dd,  $J = 4.0, 16$  Hz, 1H), 2.79 (dd,  $J = 4.0, 16$  Hz), 2.50 (t,  $J = 8.0$  Hz, 2 H), 2.22-2.32 (m, 1H), 2.00-2.12, (m, 1H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{D}_2\text{O}$ )  $\delta$  177.1, 176.8, 174.6, 71.4, 66.9, 38.5, 28.7; HRMS (ESI)  $m/z$  calcd. for  $\text{C}_9\text{H}_{14}\text{NO}_8$  ( $\text{M}+\text{H}$ ) $^+$  264.0714, found 264.0710.



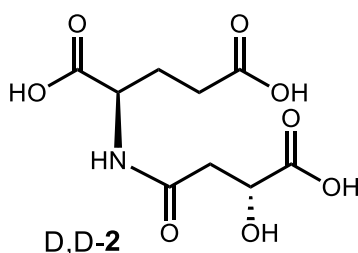
**(*R*)-2-((*S*)-3-Carboxy-2-hydroxypropanamido)pentanedioic acid (**L,D-1**)**

Prepared according to General Procedure B using **L,D-10** (63.0 mg, 0.118 mmol) to afford **L,D-1** (22.0 mg, 71% yield). IR (film) 3396, 2844, 1710, 1450, 1361,  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$  4.57 (dd,  $J = 4.0, 8.0$  Hz, 1H), 4.48 (dd,  $J = 4.0, 8.0$  Hz, 1H), 2.90 (dd,  $J = 4.0, 16$  Hz, 1H), 2.77 (dd,  $J = 4.0, 16$  Hz), 2.50 (t,  $J = 8.0$  Hz, 2 H), 2.22-2.32 (m, 1H), 2.04-2.12, (m, 1H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{D}_2\text{O}$ )  $\delta$  177.1, 176.7, 174.6, 133.7, 127.1, 71.4, 66.9, 38.6, 28.8; HRMS (ESI)  $m/z$  calcd. for  $\text{C}_9\text{H}_{14}\text{NO}_8$  ( $\text{M}+\text{H}$ ) $^+$  264.0714, found 264.0719.



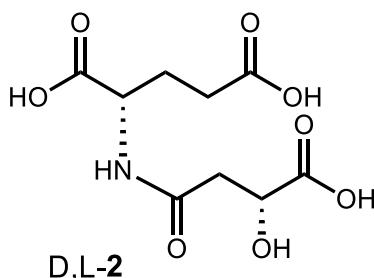
**(*S*)-2-((*S*)-3-Carboxy-3-hydroxypropanamido)pentanedioic acid (**L,L-2**)**  
(Correct structure of natural L,L-malyglutamate)

Prepared according to General Procedure B using **L,L-S1** (20.0 mg, 0.038 mmol) to afford **L,L-2** (9.0 mg, 82% yield). IR (film) 3352, 3034, 1733, 1536, 1386,  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$  4.60 (dd,  $J = 4.5, 7.8$  Hz, 1H), 4.46 (dd,  $J = 4.7, 8.2$  Hz, 1H), 3.20-3.33 (m, 1H), 2.82 (dd,  $J = 4.5, 15.2$ , 1H), 2.70-2.77 (m, 1H), 2.35-2.55 (m, 3H), 2.15-2.34 (m, 1H), 1.90-2.05 (m, 1H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{D}_2\text{O}$ )  $\delta$  177.2, 175.3, 174.9, 174.5, 67.9, 66.9, 57.5, 51.9, 48.9, 38.6, 29.9, 16.8; HRMS (ESI)  $m/z$  calcd for  $\text{C}_9\text{H}_{14}\text{NO}_8$  ( $\text{M}+\text{H}$ ) $^+$  264.0714, found 264.0721. The specific rotation  $[\alpha]_{\text{D}}^{24} = -13.6^\circ$  ( $c$  0.1, MeOH).



**(*R*)-2-((*R*)-3-Carboxy-3-hydroxypropanamido)pentanedioic acid (D,D-2)**

Prepared according to General Procedure B using **D,D-S1** (20.0 mg, 0.038 mmol) to afford **D,D-2** (8.0 mg, 80% yield). IR (film) 3352, 3034, 1733, 1536, 1386,  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$  4.60 (dd,  $J = 4.5, 7.8$  Hz, 1H), 4.46 (dd,  $J = 4.7, 8.2$  Hz, 1H), 3.20-3.33 (m, 1H), 2.82 (dd,  $J = 4.5, 15.2$ , 1H), 2.70-2.77 (m, 1H), 2.35-2.55 (m, 3H), 2.15-2.34 (m, 1H), 1.90-2.05 (m, 1H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{D}_2\text{O}$ )  $\delta$  177.2, 175.3, 174.9, 174.5, 67.9, 66.9, 57.5, 51.9, 48.9, 38.6, 29.9, 16.8; HRMS (ESI)  $m/z$  calcd for  $\text{C}_9\text{H}_{14}\text{NO}_8$  ( $\text{M}+\text{H}$ ) $^+$  264.0714, found 264.0718.



**(*S*)-2-((*R*)-3-Carboxy-3-hydroxypropanamido)pentanedioic acid (D,L-2)**

Prepared according to General Procedure B using **D,L-S1** (10.0 mg, 0.019 mmol) to afford **D,L-2** (3.8 mg, 78% yield). IR (film) 3350, 2853, 1730, 1260, 1170,  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$  4.59 (dd,  $J = 4.4, 7.8$  Hz, 1H), 4.46 (dd,  $J = 5.1, 9.0$  Hz, 1H), 2.83 (dd,  $J = 4.5, 15.2$ , 1H), 2.77 (dd,  $J = 7.9, 15.2$ , 1H), 2.51 (t,  $J = 7.5$ , 2H), 2.15-2.28;  $^{13}\text{C}$  NMR (100 MHz,  $\text{D}_2\text{O}$ )  $\delta$  177.1, 175.3, 174.8, 174.5, 67.9, 57.4, 51.7, 38.5, 29.9, 25.6, 16.7; HRMS (ESI)  $m/z$  calcd for  $\text{C}_9\text{H}_{14}\text{NO}_8$  ( $\text{M}+\text{H}$ ) $^+$  264.0714, found 264.0720.

### NMR Acquisition and Data Processing for Earthworm Samples.

$^1\text{H}$  survey spectra were acquired with 1D NOESY (noesypr1d) pulse sequence with presaturation during the 120 ms mixing time. Spectra were acquired at 25  $^\circ\text{C}$  with 64 scans, 16 dummy scans, 2 s delay time, 1.95 s acquisition time, 13.9848 ppm spectra width, and 32768 points. The FIDs were exported into MestReNova 12 (Santiago de Compostela, Spain) and apodized by multiplication with an exponential function equivalent to 0.5 Hz line broadening, and zero-filled to 131072 points prior to Fourier transformation. The resulting spectra were manually phased and referenced to DSS- $d_6$  (0 ppm).

The HMBC (hmbcgp1pndqf) experiment was conducted using an 80-worm pooled coelomocyte sample acquired at 25  $^\circ\text{C}$  with 64 scans, 16 dummy scans, a  $^1\text{H}$  90 $^\circ$  pulse of 13.25  $\mu\text{s}$ , a  $^{13}\text{C}$  90 $^\circ$  pulse of 13.00  $\mu\text{s}$ , a 65 ms evolution delay, a proton-carbon coupling constant ( $^nJ_{\text{CH}}$ ) of 10 Hz, and a 2 s relaxation delay. Spectral widths of 13.9848 ppm and

210.0000 ppm were used with 2048 by 512 complex points in F2 and F1, respectively. The HMBC spectrum was apodized using a sine function, baseline corrected with a third order polynomial in both dimensions, smoothed using a Savitzky-Golay filter with an order of 4 and width of 16 in both dimensions, zero-filled to 4096 by 4096 points, and manually phased.

## References

- (1) Chan, T. M.; Kong, J.; McNamara, P.; Wong, J. K., *Synth. Commun.* **2008**, 38 (13), 2252-2260.
- (2) Du, Z.; Lu, Y.; Dai, X.; Zhang-Negrerie, D.; Gao, Q., *J. Chem. Res.* **2013**, 37 (3), 177-180.
- (3) Schobert, R.; Jagusch, C., *Synthesis* **2005**, 14, 2421-2425
- (4) Dutton, F. E.; Lee, B. H., *Tetrahedron Lett.* **1998**, 39, 531



# NMR Spectra

