Electronic supporting information (ESI) Biosynthesis of Isoxazolin-5-one and 3-Nitropropanoic acid Containing Glucosides in Juvenile Chrysomelina

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Overview of the syntheses

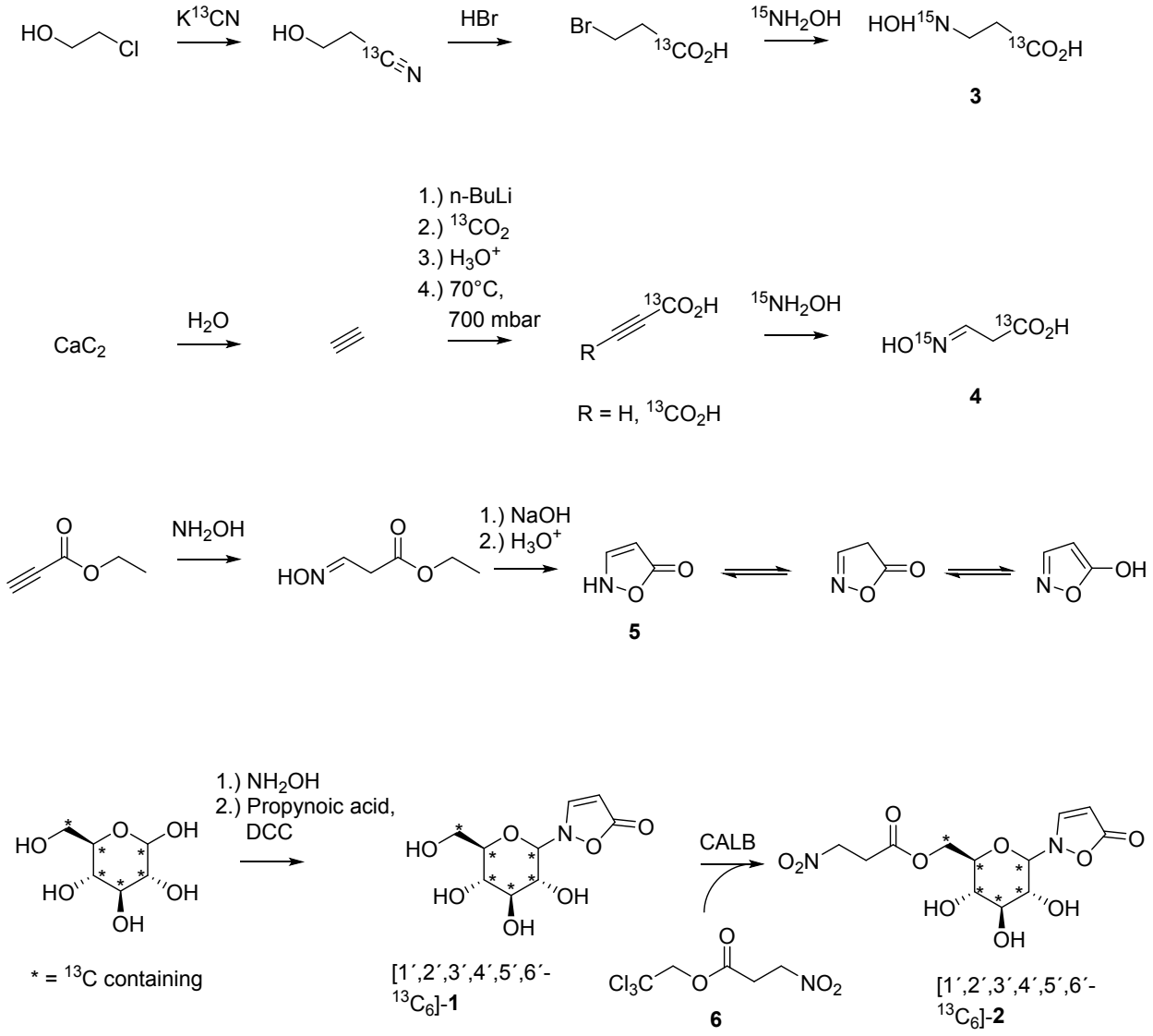


Fig. S1: Synthetic routes for the described substrates **1** to **5**.

Overview of the incorporation results

Table S1:

Compound	Significance of incorporation
[$^{13}C_5{}^{15}N$]-Val	***
[$^{13}C_3$]-propanoate	***
[$^{13}C_3{}^{15}N$]-β-Ala	***
[1-$^{13}C^{15}N$]-3-(hydroxyamino)propanoic acid **3**	***
[1-$^{13}C^{15}N$]-3-(hydroxyimino)propanoic acid **4**	***
isoxazolin-5-one **5** [a]	*** [a]
[1-$^{13}C^{15}N$]-3-nitropropanoic acid [b]	*** [b]
[$^{13}C_4{}^{15}N$]-Asp	-
[4-^{13}C]-Asp	-
[$^{13}C_4$]-Asp	-
[$^{13}C_4{}^{15}N$]-Thr	-
[$^{13}C_2$]-malonate	-
[$^{13}C_3{}^{15}N$]-α-L-Ala [c]	- [c]
[^{15}N]-α-L-Ala [c]	- [c]

"-" = no significant intact incorporation into compounds **1** and **2** observed

[a] *in vitro* assays

[b] shown *in vivo* in a previous study: G. Pauls, T. Becker *et al*. as well as *in vitro* in this study using unlabeled **1**, **5**, ATP, CoA as well as 3-NPA

[c] Only Nitrogen incorporation

Synthetic protocols and spectra

[1-^{13}C, ^{15}N]-3-(hydroxyamino)propanoic acid **3**

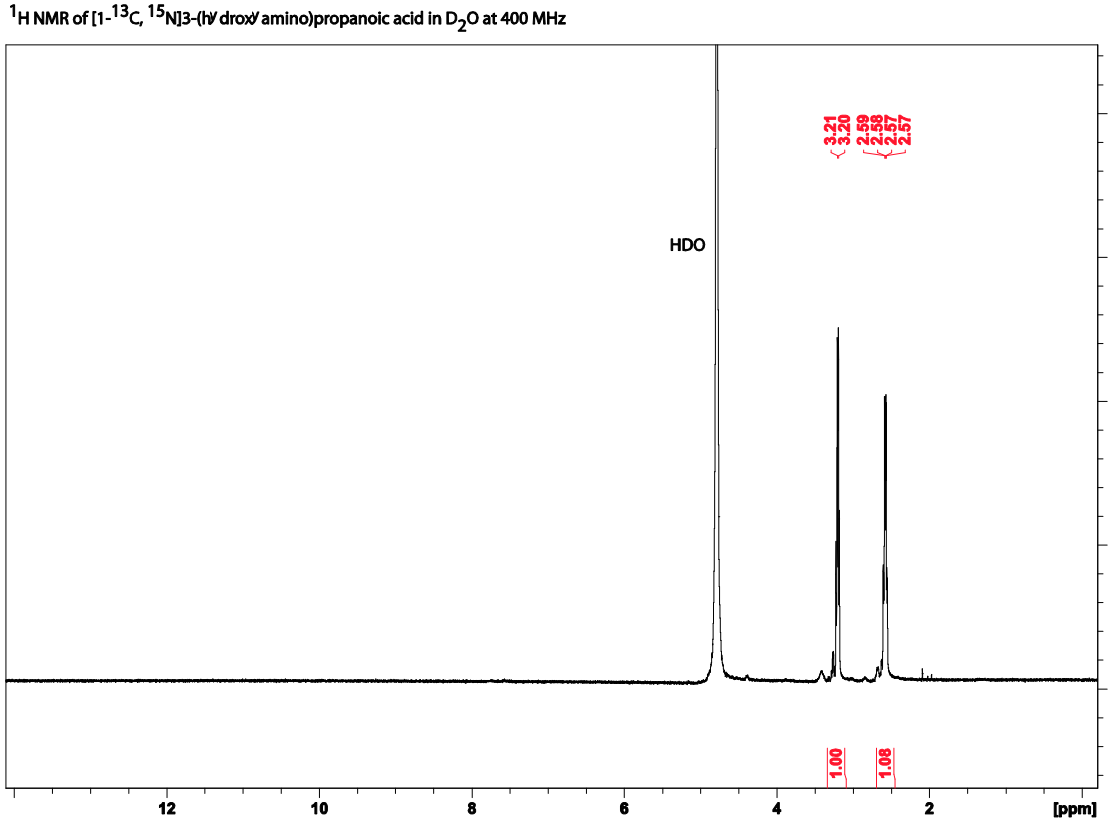
2.223 g (27.61 mmol) of 2-chloroethanol were dissolved in 13.1 ml ethanol and 6.5 ml water. Then 1.034 g (6.9 mmol) NaI and 661 mg (10 mmol) K^{13}CN were added. The mixture was heated to 70 °C for 18h. After the reaction time was finished the solvents were removed at 40 °C under reduced pressure and the residual oil was taken up with ethyl acetate (5 ml). The mixture was added to 5g of dry silica and eluted with ethyl acetate (30 ml). The solvent was removed to obtain 1.9 g (26.4 mmol, 95.5%) 3-hydroxypropionitrile. The intermediate was dissolved in 25.5 ml of HBr (40% in water) and the mixture was heated for 2.5 h. Then 20 ml water were added and the mixture was extracted with diethyl ether (7 x 50 ml). The combined organic phases were dried over $MgSO_4$, filtrated and the solvent was removed at 40 °C under reduced pressure to obtain 840 mg (5.456 mmol, 20.7%) 3-bromopropanoic acid. The product was dissolved in diethyl ether (10 ml), a solution of 216.3 mg (6.55 mmol) NH_2OH in MeOH as well as 377 mg (2.728 mmol) K_2CO_3 were added. NH_2OH in methanol was prepared from 461.5 mg (6.55 mmol) NH_2OH*HCl that was dissolved in dry methanol (6.6 ml). To the solution of NH_2OH*HCl 704.1 mg (6.274 mmol) KOtBu were added at 0 °C. After 15 min of stirring at rt the mixture was filtrated and washed with dry methanol (3 x 1.1 ml). The mixture of 3-bromopropanoic acid, NH_2OH and K_2CO_3 in MeOH/Et_2O was stirred for 18h at 40 °C. The solvents were removed at 40 °C under reduced pressure, the residue was taken up in MeCN/H_2O (3:1) and eluted with this eluent over SiO_2. The solvents were removed from the product fractions to obtain a colorless solid **3** (20 mg, 3.5%).

R_f(MeCN/H_2O 3:1)=0.16;

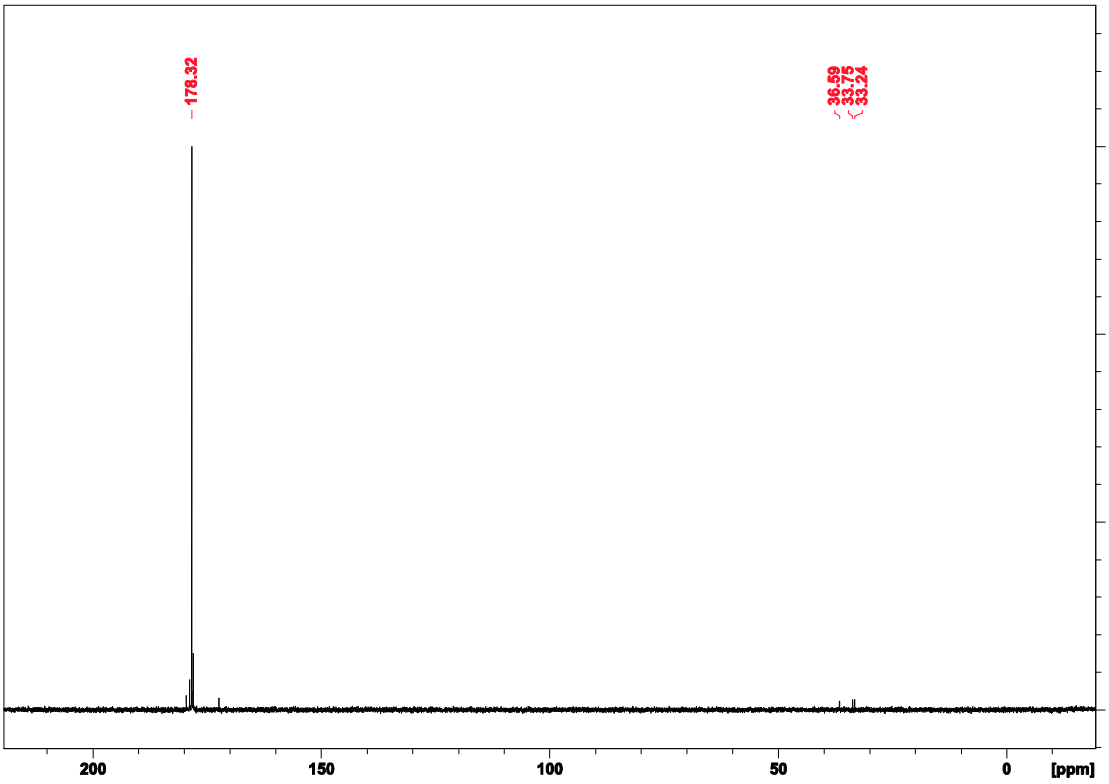
^{1}H NMR (400 MHz, $CDCl_3$) δ 3.21 (q, J = 5.2 Hz, 2H, H-3), 2.58 (dq, J_1 = 6.5 Hz, J_2 = 3.0 Hz, 2H, H-2);

^{13}C NMR (100 MHz, $CDCl_3$) δ 178.3 (s, C-1), 36.6 (d, $^{1}J_{15N13C}$ = 5.7 Hz, C-3), 33.5 (d, $^{1}J_{1,2}$ = 50.8 Hz, C-2);

HRMS (ESI-TOF) m/z calcd for $C_2{}^{13}CH_6{}^{15}NO_3^-$ 106.035706 [M - H]$^-$, found 106.035769.



^{13}C NMR of [1-^{13}C, ^{15}N]3-(hydroxyamino)propanoic acid in D_2O at 100 MHz



[1-^{13}C, ^{15}N]-3-(hydroxyimino)propanoic acid **4**

At 0 °C water was added slowly and dropwise to 10g CaC_2. The developing acetylene was dried with $CaCl_2$ and lead through a solution of 15 ml n-BuLi (c = 1.5 mol/l, n = 22.5 mmol) in 20 ml of dry THF under argon atmosphere at 0 °C. After precipitation of a colorless solid $^{13}CO_2$ was lead through the solution for 20 h at 0 °C to rt. Then the solution was neutralized with 3.4 ml of HCl in water (3.18 mol/l) and 5 ml KOH in water (1 mol/l) were added. The mixture was heated to 70 °C under reduced pressure (700 mbar) for 2h. Then 15 ml of HCl in water (3.18 mol/l) were added and the mixture was extracted with diethyl ether (5 x 100 ml). The solvents of the combined organic phases were removed at 40 °C and reduced pressure to yield crude [1-^{13}C]propynoic acid as a yellow oil (1.05 g). The crude product was added to a solution of $^{15}NH_2OH$ in dry methanol, that was prepared by dissolving $^{15}NH_2OH$*HCl (534 mg, 7.58 mmol) in 8 ml of dry methanol, addition of KOtBu (2.52 g, 22.5 mmol) at 0 °C, filtration of the solution and washing with 4 ml of dry methanol. After stirring for 4d at rt the mixture was concentrated to 2 ml, added to a column (Silica) and eluted with EtOAc/MeOH/AcOH 100:10:1. After removal of the eluent from the product fractions at 40 °C and reduced pressure a colorless solid **4** was obtained (57 mg, 0.54 mmol, 7.2%).

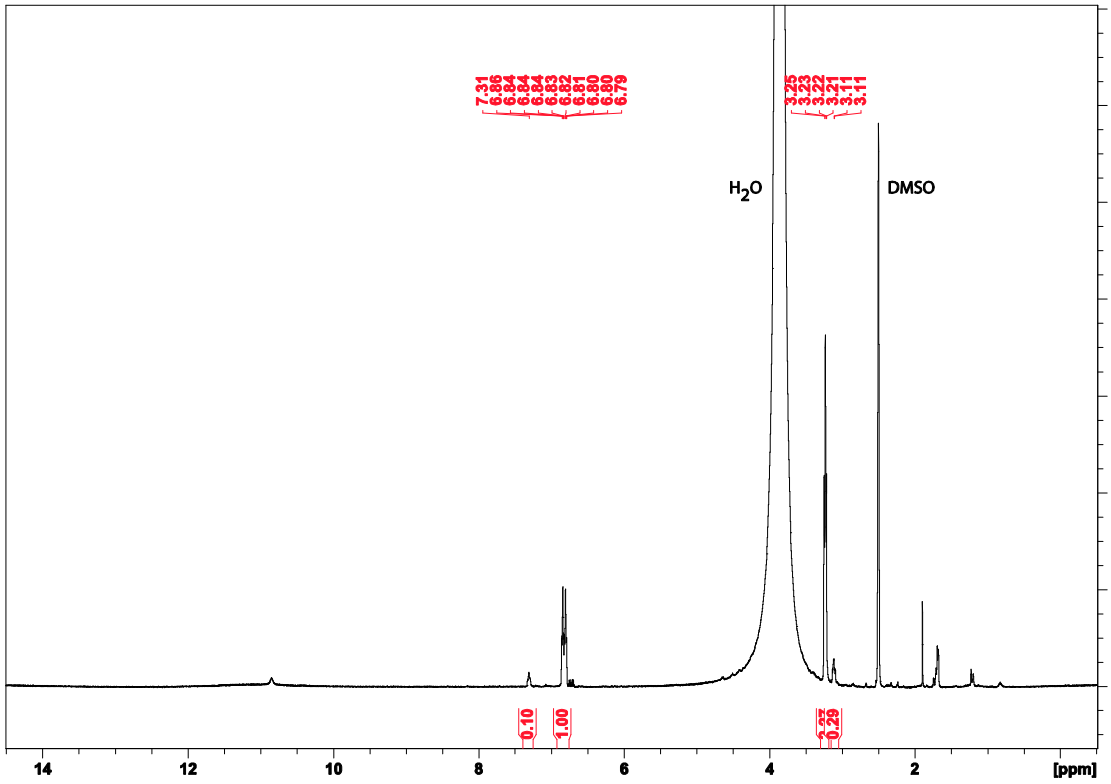
R_f(EtOAc/MeOH/AcOH 100:10:1)=0.63;

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 (m, 0.1H, *Z*-H-3), 6.82 (m, 1H, *E*-H-3), 3.23 (m, 2H, *E*-H-2), 3.11 (m, 0.29H, *Z*-H-2);

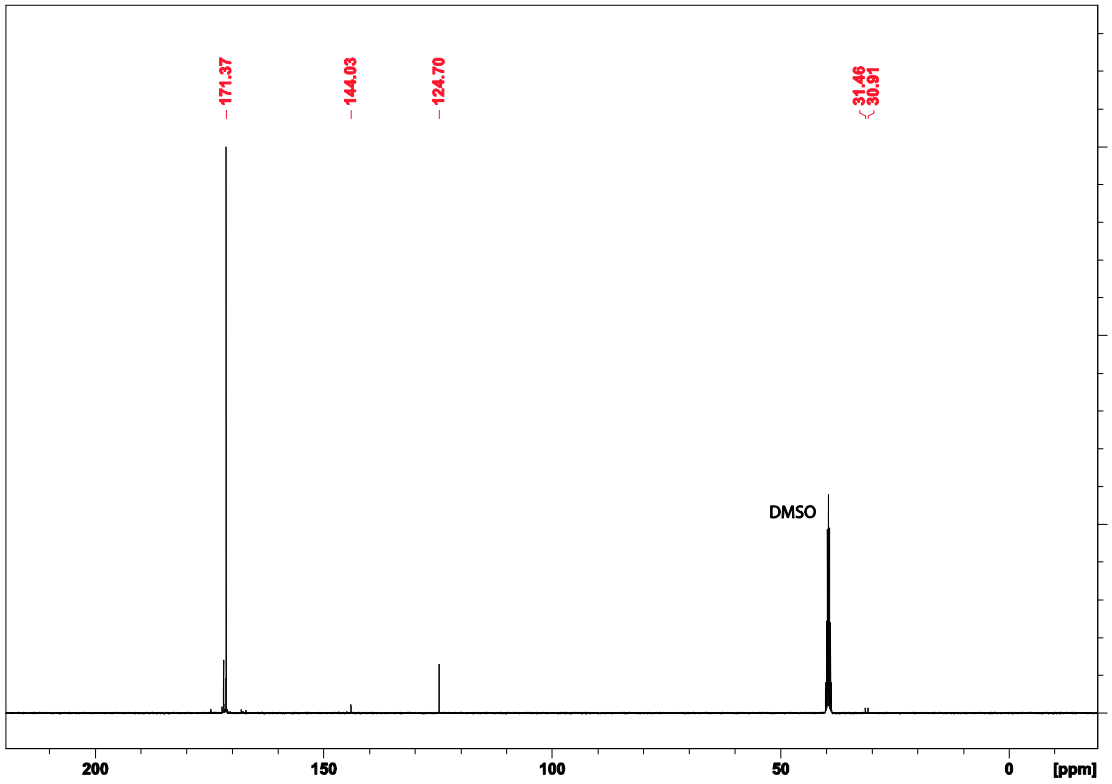
^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4 (s, C-1), 144.0 (s, *Z*-C-3), 124.7 (s, *E*-C-3), 31.2 (d, $^1J_{1,2}$ = 55.7 Hz, C-2);

HRMS (ESI-TOF) m/z calcd for $C_2{}^{13}CH_4{}^{15}NO_3^-$ 104.02006 $[M - H]^-$, found 104.020056.

^{1}H NMR of [1-^{13}C, ^{15}N]3-(hydroxyimino)propanoic acid in DMSO at 400 MHz



^{13}C NMR of [1-^{13}C, ^{15}N]3-(hydroxyimino)propanoic acid in DMSO at 100 MHz



Isoxazolin-5-one **5**

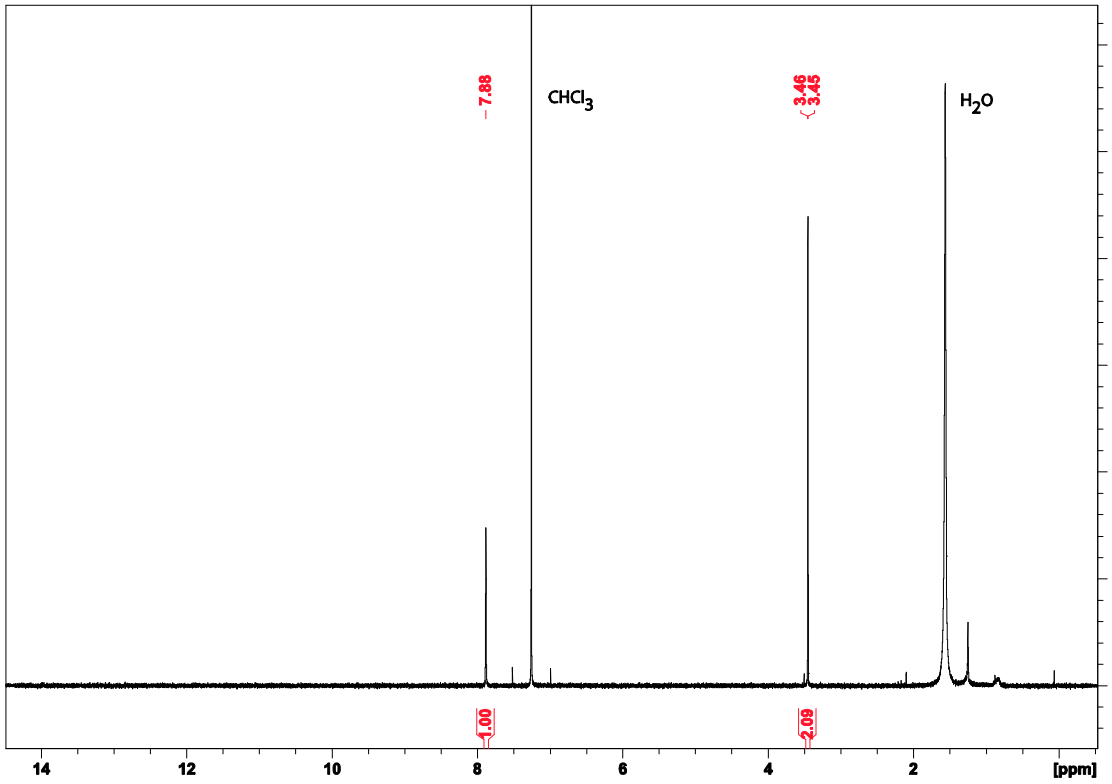
6.57 g (94.6 mmol) NH_2OH*HCl were added at 0 °C to a solution of 21.1 ml KOH in water (c = 4.49 mol/l). Then 4.64 g (47.3 mmol) ethyl propiolate in 37.8 ml ethanol and 9.47 g (94.46 mmol) $KHCO_3$ were added to the mixture at 0 °C. After stirring for 20h at -15 to 7 °C 100 ml of water were added. The mixture was extracted with diethyl ether (3 x 80 ml). The combined organic phases were counter extracted with water (1 x 100 ml). The organic phase was dried over $MgSO_4$, filtrated and the solvent was removed at 40 °C under educed pressure to yield ethyl 3-(hydroxyimino)propanoate as a colorless solid (2.78 g, 21.2 mmol, 44.8%). Without further characterization the oxime (2.78 g, 21.2 mmol) was dissolved in 53 ml water and cooled to 0 °C. Then 21.5 ml of a solution of NaOH in water (c = 4.49 mol/l) was added. The mixture was stirred for 10 min and acidified with 35.2 ml of HCl in water (c = 3.18 mol/l)and extracted with diethyl ether (3 x 100 ml). The combined organic phases were dried with $MgSO_4$, filtrated and the solvent was removed at 40 °C under reduced pressure to yield a yellow powder (1.51 g, 17.8 mmol, 83.7%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (m, 1H, H-3), 3.46 (d, $^3J_{3,4}$ = 1.4 Hz, 2H, H-4);

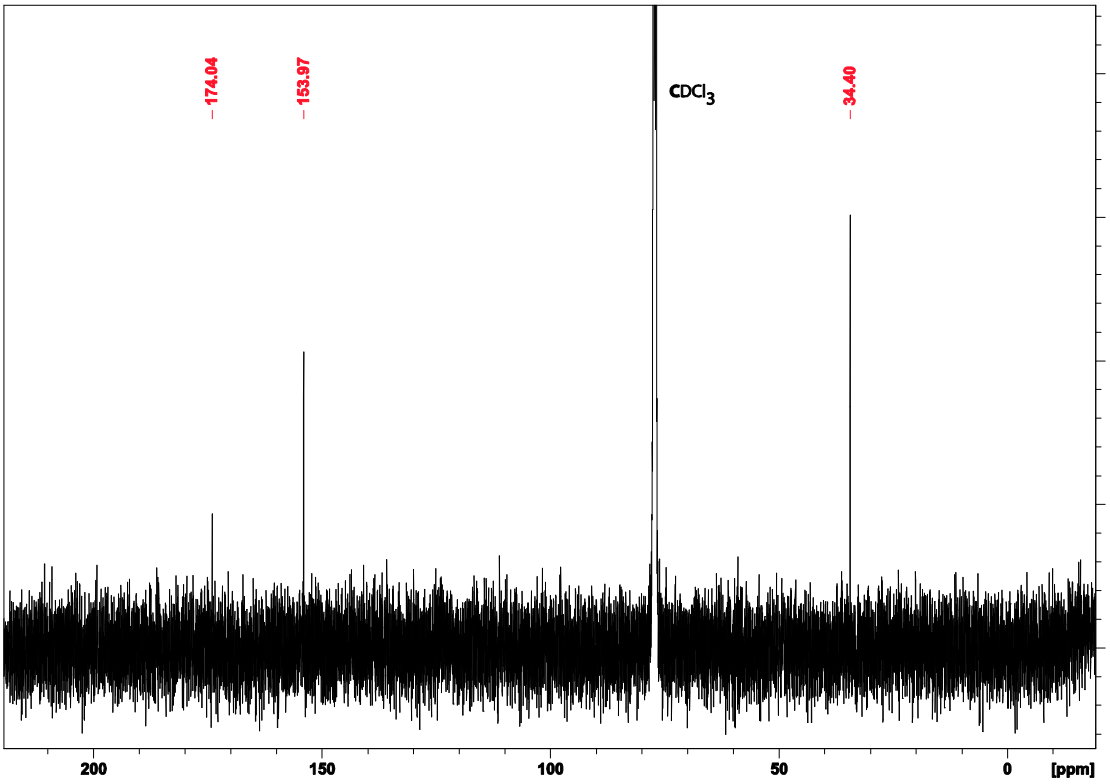
^{13}C NMR (100 MHz, $CDCl_3$) δ 174.0 (C-5), 154.0 (C-3), 34.4 (C-4);

HRMS (ESI-TOF) m/z calcd for $C_3H_2NO_2^-$ 84.00910 $[M - H]^-$, found 84.00928;

^{1}H NMR of Isoxazolin-5(2*H*)-one in $CDCl_3$ at 400 MHz



^{13}C NMR of Isoxazolin-5(2*H*)-one in $CDCl_3$ at 100 MHz



[1′,2′,3′,4′,5′,6′-$^{13}C_6$]-2-(β-D-Glucopyranosyl)-3-isoxazolin-5-one [1′,2′,3′,4′,5′,6′-$^{13}C_6$]-**1**

To a stirred solution of 581 mg (8.362 mmol, 1.5 eq.) hydroxylamine hydrochloride in 8 ml dry methanol 875 mg (7.798 mmol, 1.4 eq.) potassium tert-butoxide were added in 5 portions at 0 °C under stirring. After 15 min at rt the solution was filtered under vacuum, washed with 4 ml of dry methanol and 1.0375g (5.57 mmol) of [1′,2′,3′,4′,5′,6′-$^{13}C_6$]glucose was added. After 1 d of stirring at rt the solvent was removed under reduced pressure at 40 °C. The dry residue was dissolved in 4 ml of water. Under stirring 20 ml of DCC in MeCN (c = 0.4 M, 1.44 eq.) and 19.5 ml of propynoic acid in MeCN (c = 0.42 M, 1.47 eq.) were added simultaneously at rt over 5 h. After 20 h of stirring at rt the solvents were removed at 25 °C under reduced pressure. The mixture was taken up in 10 ml of water and applied to an ultrasound bath for 1 h at 22–27 °C. The suspension was filtrated and washed with water (3 × 10 ml). To the filtrate 500 ml of MeCN were added and the solvents were removed at 25 °C and 200 to 75 mbar. Then 1.25 g of dry silica and 250 ml MeCN were added. The solvents were removed again at 25 °C and 200 to 75 mbar to yield a dry crude mixture. The dry mixture was applied to a column and eluted (MeCN/H2O 55 : 1, silica). The product fractions were combined and concentrated to yield 250 mg (0.973 mmol, 17.5 %) of [1′,2′,3′,4′,5′,6′-$^{13}C_6$]**1** as a colorless powder.

R_f(MeCN/H_2O 55:1)=0.15

^{1}H NMR (400 MHz, D_2O) δ 8.45 (d, $^3J_{3,4}$ = 3.7 Hz, 1H, H-3), 5.47 (d, $^3J_{3,4}$ = 3.7 Hz, 1H, H-4), 5.34-4.89 (m, 1H, H-1′), 4.12-3.26 (m, 6H, H-2′to H-6′);

^{13}C NMR (100 MHz, D_2O) δ 175.3 (s, C-5), 155.4 (s, C-3), 91.2 (s, C-4), 88.9 (dt, J_1 = 43.3 Hz, J_2 = 4.3 Hz, C-1′), 78.8 (t, J = 41.5 Hz, C-5′), 76.8 (t, J = 39.4 Hz, C-3′), 71.0-69.6 (m, C-2′ and C-4′); 61.0 (dt, J_1 = 43.0 Hz, J_2 = 3.9 Hz, C-6′);

LC/MS (RP18e MeCN/H_2O/APCI) m/z for $C_4{}^{13}C_6H_{14}NO_9^-$ 298.1 $[M + FA - H]^-$.

[1′,2′,3′,4′,5′,6′-$^{13}C_6$]-2-[6′-(3″-Nitropropanoyl)-β-D-glucopyranosyl]-3-isoxazolin-5-one [1′,2′,3′,4′,5′,6′-$^{13}C_6$]-**2**

A mixture of 60 mg (0.237 mmol) 2-(β-D-glucopyranosyl)-3-isoxazolin-5-one **1**, 94 mg (0.375 mmol) 2,2,2-trichloroethyl 3-nitropropanoate **6**, 90 mg immobilized *C. antarctica* lipase B and 4 Å molecular sieves was suspended in 4.2 ml dry tert-butyl alcohol. The suspension was stirred at 50 °C under an argon atmosphere for 3d. 0.65 g of dry silica was added and the mixture was concentrated under reduced pressure at 40 °C. The dry residue was added to a silica column, and the product was purified by column chromatography (ethyl acetate/MeOH/DCM 10:1:1 to 2:1:0). The solvent was removed to yield **2** as a colorless solid (15.6 mg, 0.044 mmol, 18.5 %). Nonconverted glucoside [1′,2′,3′,4′,5′,6′-$^{13}C_6$]**1** could be recovered (15 mg, 0.059 mmol, 25 %).
R_f(ethyl acetate/MeOH/DCM 10:1:1)=0.20

^{1}H NMR (400 MHz, D_2O) δ 8.46 (d, $^3J_{3,4}$ = 3.7 Hz, 1H, H-3), 5.50 (d, $^3J_{3,4}$ = 3.7 Hz, 1H, H-4), 5.35-4.80 (m, 1H, H-1′), 4.57-3.09 (m, 10H, H-2′ to H-3′′);

^{13}C NMR (100 MHz, D_2O) δ 174.7 (C-5), 172.4 (C-1″), 155.0 (C-3), 91.7 (C-4), 88.7 (dt, J_1 = 43.4 Hz, J_2 = 4.3 Hz, C-1′), 77.0-75.6 (m, C-5′ and C-3′), 70.4-69.0 (m, C-3″, C-2′ and C-4′), 63.9 (dt, J_1 = 44.2 Hz, J_2 = 4.1 Hz, C-6′), 31.7 (C-2″);
LC/MS (RP18e MeCN/H_2O/APCI) m/z for $C_7{}^{13}C_6H_{17}N_2O_{12}^-$ 398.9 $[M + FA - H]^-$.

2,2,2-Trichloroethyl 3-Nitropropanoate **6**

3-Nitropropanoic acid (687 mg, 5.77 mmol), 2,2,2-trichloroethanol (3.45 g, 23.08 mmol, 4 equiv), and DMAP (63.4 mg, 0.52 mmol, 9 mol %) were dissolved in dry DCM (5.77 mL). The mixture was cooled to 0 °C, and DCC (1.308 g, 6.35 mmol, 1.1 equiv) was added all at once.

After 10 min at 0 °C, the mixture was heated to rt and stirred for 3 h. After purification by flash column chromatography (CHCl3) and removal of the solvent at 40 °C under reduced pressure, a colorless powder of **6** (834 mg, 3.33 mmol, 57.7%) was obtained.

$R_f(CHCl_3)$=0.78;

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.81 (s, 2H, CH_2CCl_3), 4.72 (t, $^3J_{2,3}$ = 6.1 Hz, 2H, CH_2NO_2), 3.16 (t, $^3J_{2,3}$ = 6.1 Hz, 2H, CH_2CO_2R);

^{13}C NMR (125 MHz, $CDCl_3$) δ 168.13, 94.46, 74.59, 69.35, 30.97; HRMS (APCI-Orbitrap) m/z calcd for $C_5H_7Cl_3NO_4$ 249.9435 $[M + H]^+$, found 249.9429;

In vitro experiments

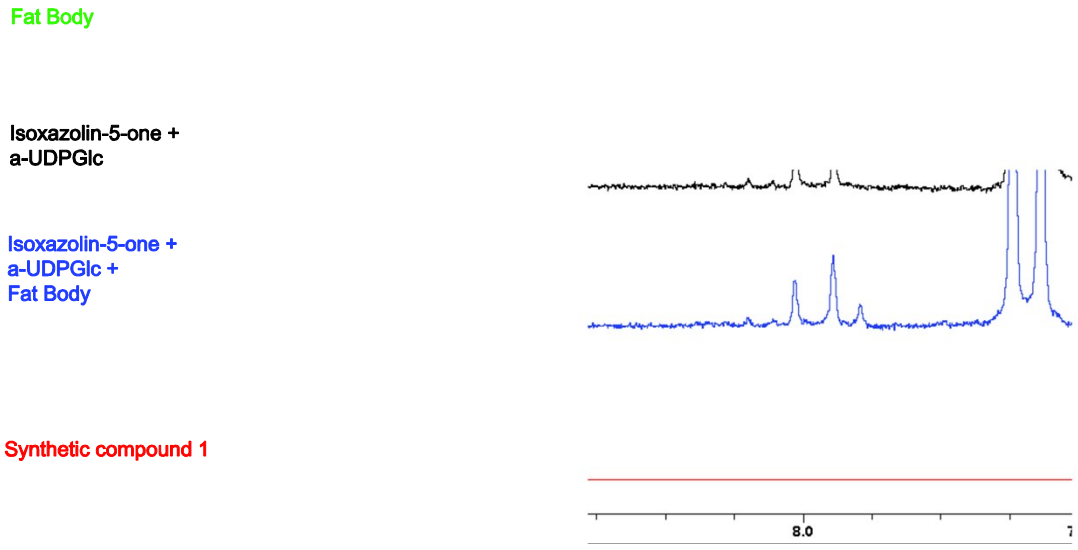


Fig. S2: Representative 400 MHz ^{1}H NMR spectra of buffered solutions of the fat body, as a control, (green) of *Phaedon cochleariae*, compound **5** and α-UDP-Glucose (black), compound **5**, α-UDP-Glucose and fat body (blue) as well as the synthetic compound **1** (red) after 1d of incubation at 30 °C.

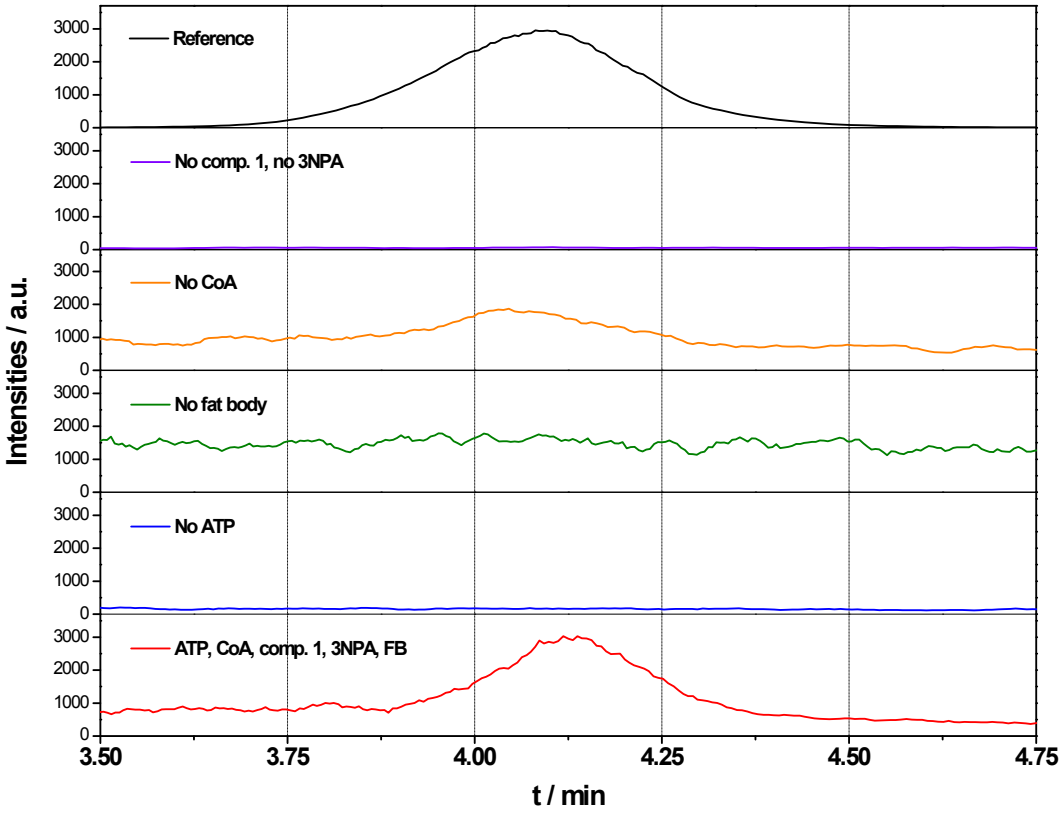
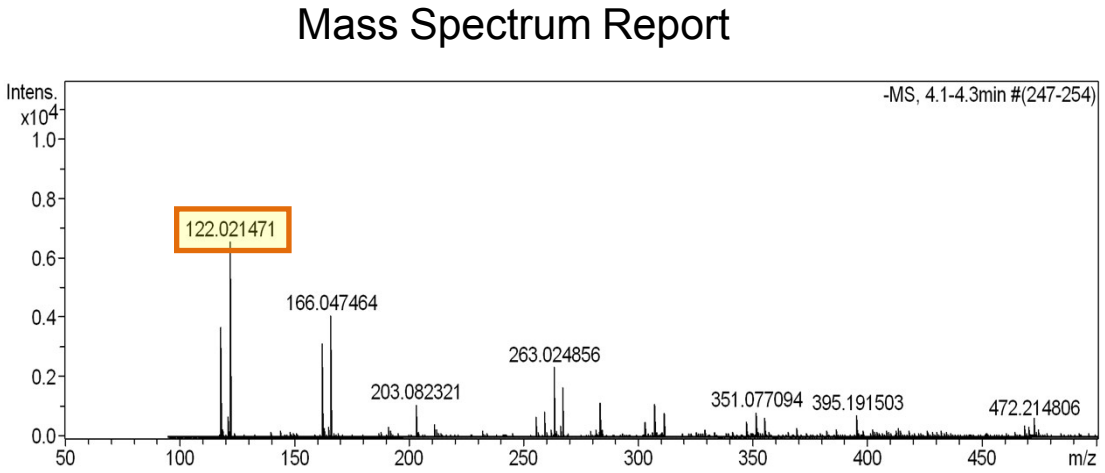


Fig. S3: Representative HPLC-MS signal of *m*/*z* 393 of buffered solutions containing ATP, CoenzymeA, compound **1**, 3-NPA and/or the fat body of *Chrysomela populi* after 1d of incubation at 30 °C.

In vivo experiments



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf
118.014285	1	C 5 N 3 O	0.00	118.004685	-9.600	-81.347	1.6	7.5	even
	2	C 4 N 5	62.83	118.015919	1.633	13.840	3.5	7.5	even
	3	C 3 H 4 N O 4	100.00	118.014581	0.296	2.507	18.9	2.5	even
	4	C 2 H 4 N 3 O 3	0.00	118.025815	11.529	97.694	20.6	2.5	even
	5	C 6 H N 2 O	0.00	117.009436	-2.127	-18.020	535.6	7.5	even
	6	C 5 H N 4	0.00	117.020670	8.390	71.095	537.6	7.5	even
	7	C 4 H 5 O 4	0.00	117.019332	8.468	71.752	552.4	2.5	even
	8	C H N 4 O 3	0.00	117.005413	-8.946	-75.801	562.6	3.5	even
121.027141	1	C 2 H 5 N 2 O 4	64.18	121.025480	-1.660	-13.720	17.4	1.5	even
122.021471	1	H 4 O 4 ^13C 3 ^15N	100.00	122.021681	0.209	1.714	7.4	2.5	even
	2	C N 4 ^13C 3 ^15N	41.71	122.023018	1.547	12.675	14.8	7.5	even
	3	C 2 N 2 O ^13C 3 ^15N	0.00	122.011785	-9.687	-79.386	20.7	7.5	even
	4	C 3 H N O ^13C 3 ^15N	0.00	121.016536	-2.200	-18.029	801.9	7.5	even
	5	C 2 H N 3 ^13C 3 ^15N	0.00	121.027769	7.529	61.705	803.3	7.5	even
124.025620	1	C 2 H 6 N O 5	100.00	124.025146	-0.474	-3.821	15.8	0.5	even
162.040609	1	C 6 H 4 N 5 O	65.55	162.042133	1.525	9.409	6.2	7.5	even

Fig. S4: Representative high resolution mass spectrum of free 3-NPA after LC separation of larval MeCN/H_2O (1:1) extracts from *P. cochleariae* upon feeding on [$^{13}C_3{}^{15}N$]-β-Ala in KH_2PO_4/K_2HPO_4 for 10 d.

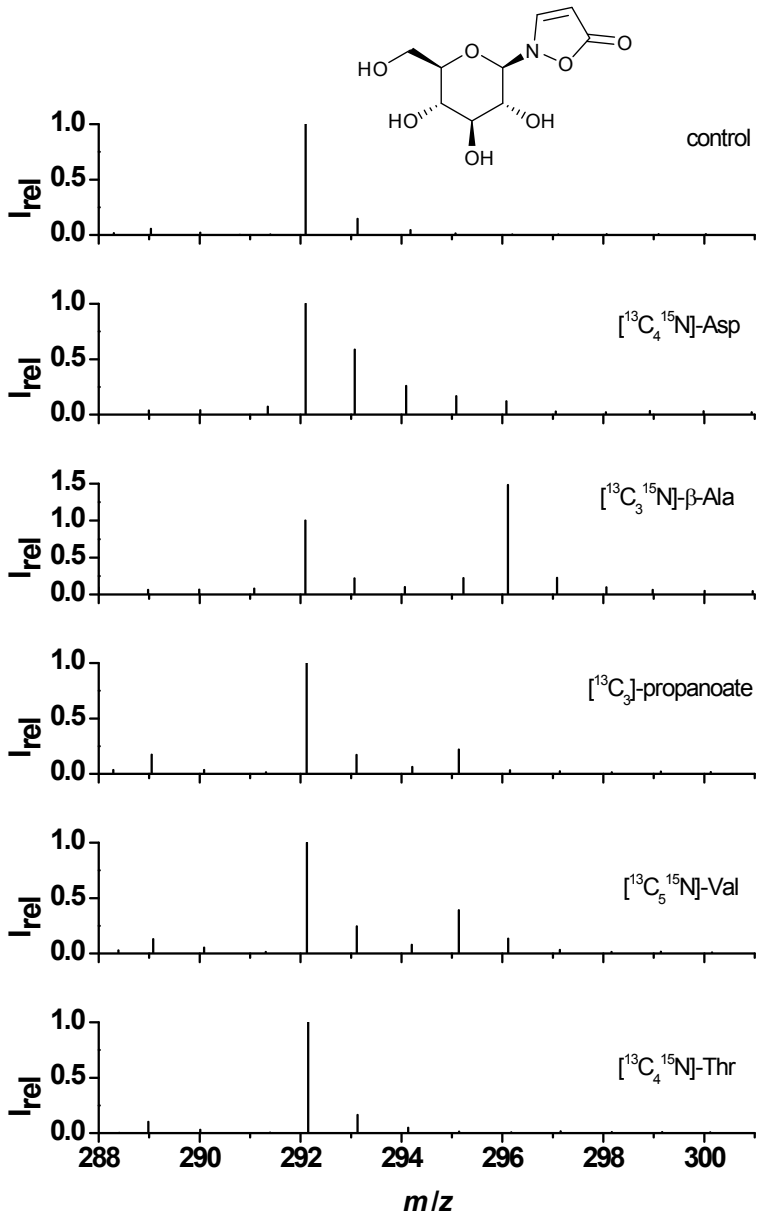


Fig. S5 Representative mass spectra of compound **1** after LC separation of larval extracts (MeCN/H_2O, 1:1) from *P. cochleariae* after feeding on different diets for 10 d; diets consisted of *B. rapa pekinensis* leaves, impregnated with KH_2PO_4/K_2HPO_4 buffered solutions of the compounds given above; as a control only buffer was used.

Quantification of compounds 1 and 2 in Chrysomelina

The quantifications were carried out via addition of $^{13}C_6$ isotopic labelled standards of compounds **1** and **2** to whole larval extracts (spiking). As shown in our previous work (G. Pauls, T. Becker *et al.*), the ester moiety hydrolyses in compound **2** upon extraction with aqueous or alcoholic media to a certain extent, so that free 3-NPA can be detected upon HPLC-MS analysis. Due to these circumstances, the quantifications were carried out as soon as possible after extraction of the samples. Furthermore the samples were stored at -25 °C prior to analysis.

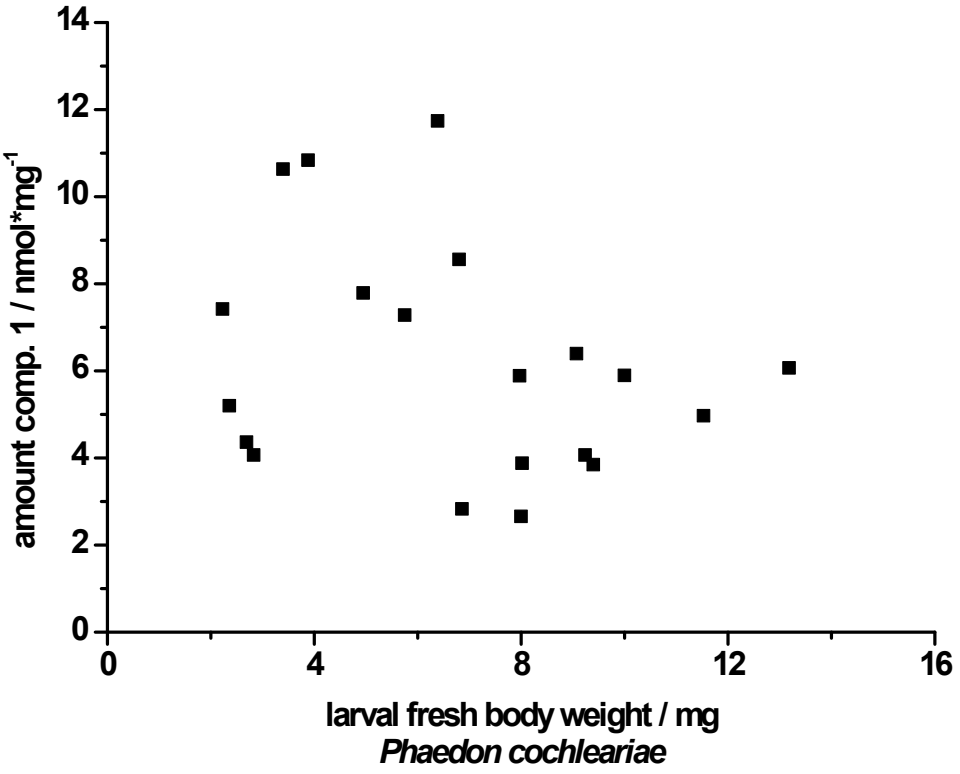


Fig. S6: Molar amount of compound **1** in *Phaedon cochleariae* per body weight, feeding on *Brassica rapa pekinensis* leafs.

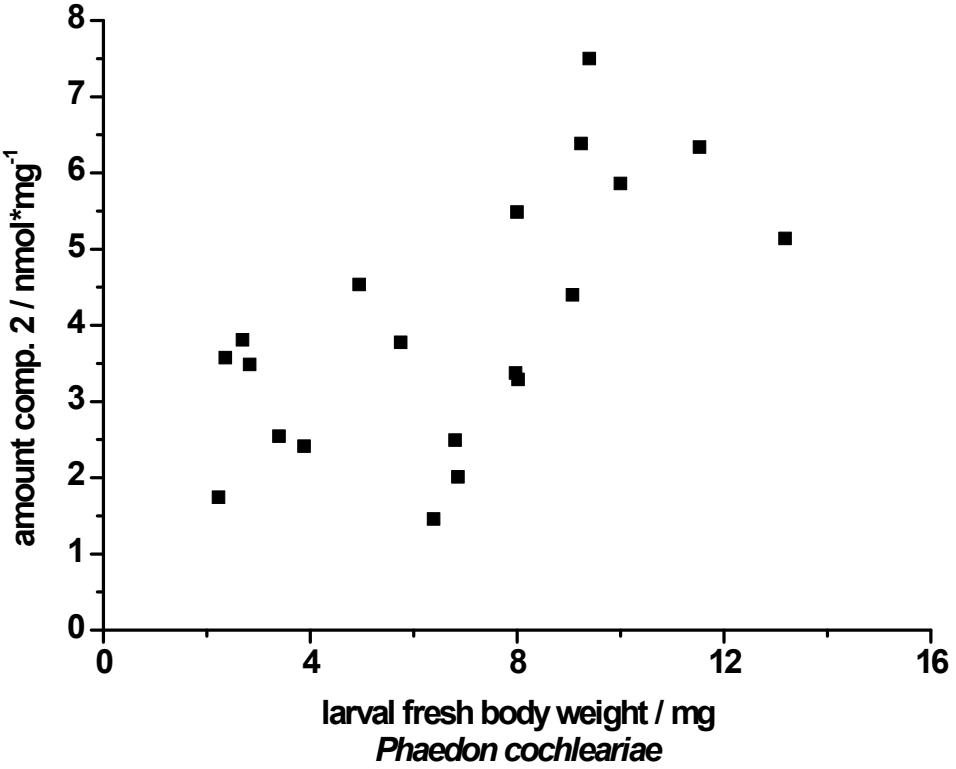


Fig. S7: Molar amount of compound **2** in *Phaedon cochleariae* per body weight, feeding on *Brassica rapa pekinensis* leafs.

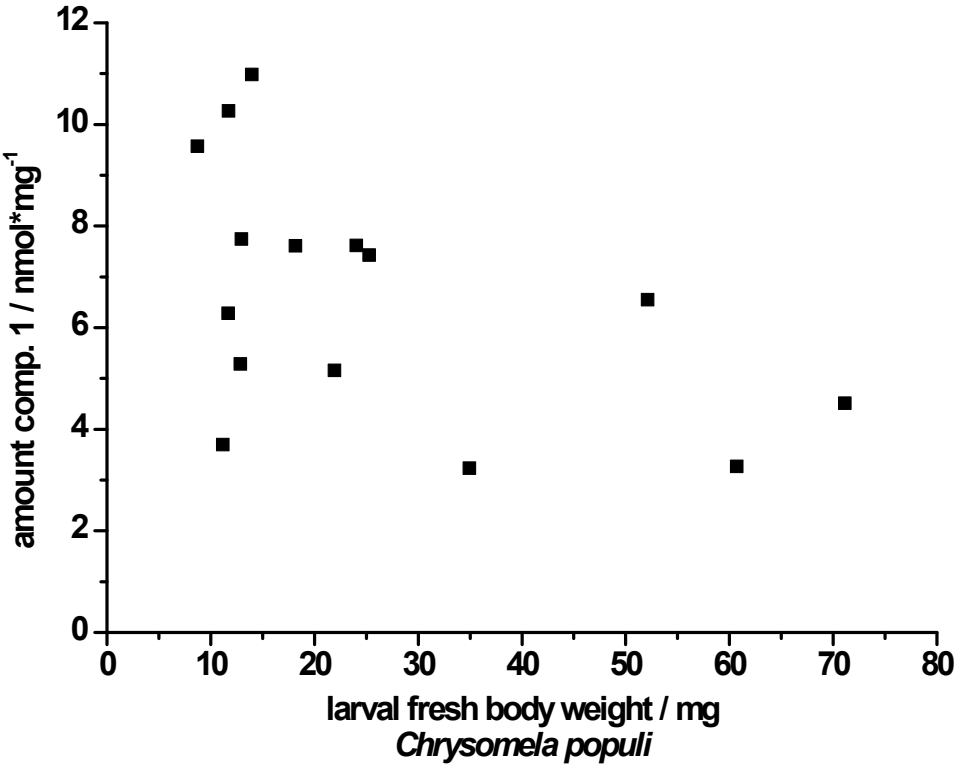


Fig. S8: Molar amount of compound **1** in *Chrysomela populi* per body weight, feeding on *Populus canadensis* leafs.

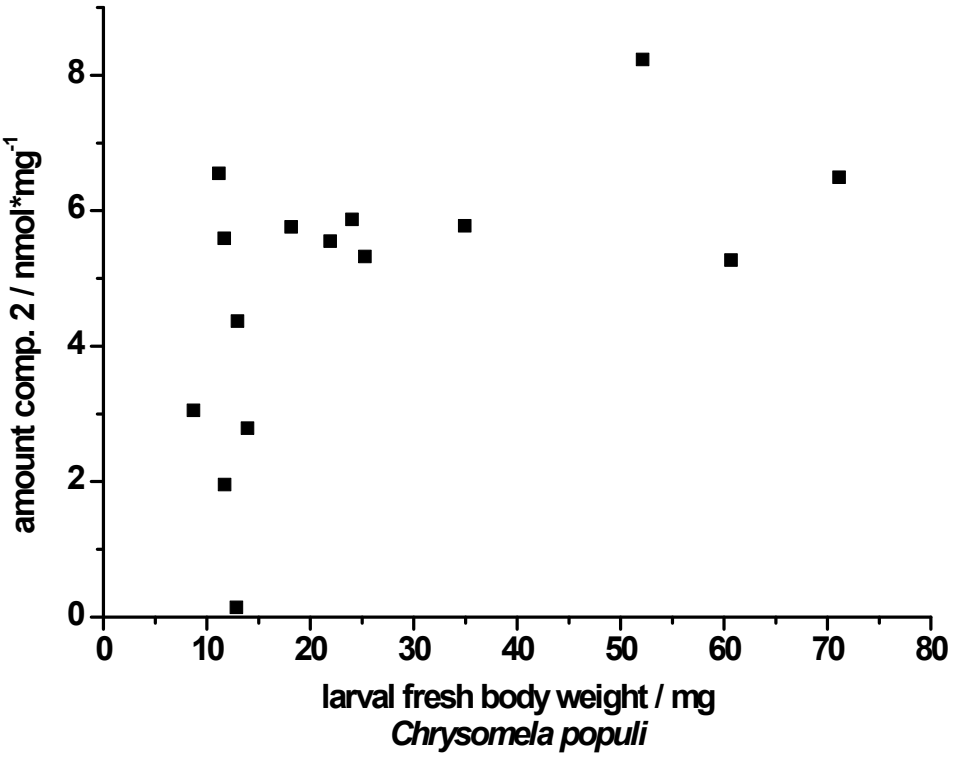


Fig. S9: Molar amount of compound **2** in *Chrysomela populi* per body weight, feeding on *Populus canadensis* leafs.

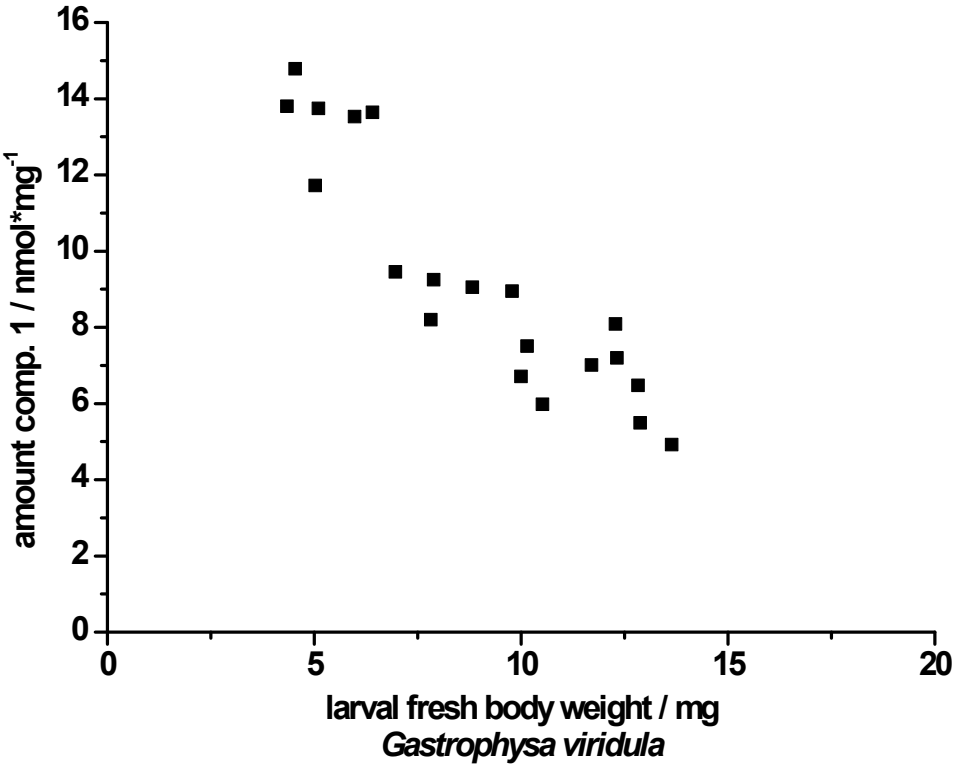


Fig. S10: Molar amount of compound **1** in *Gastrophysa viridula* per body weight, feeding on *Rumex obtusifolius* leafs.

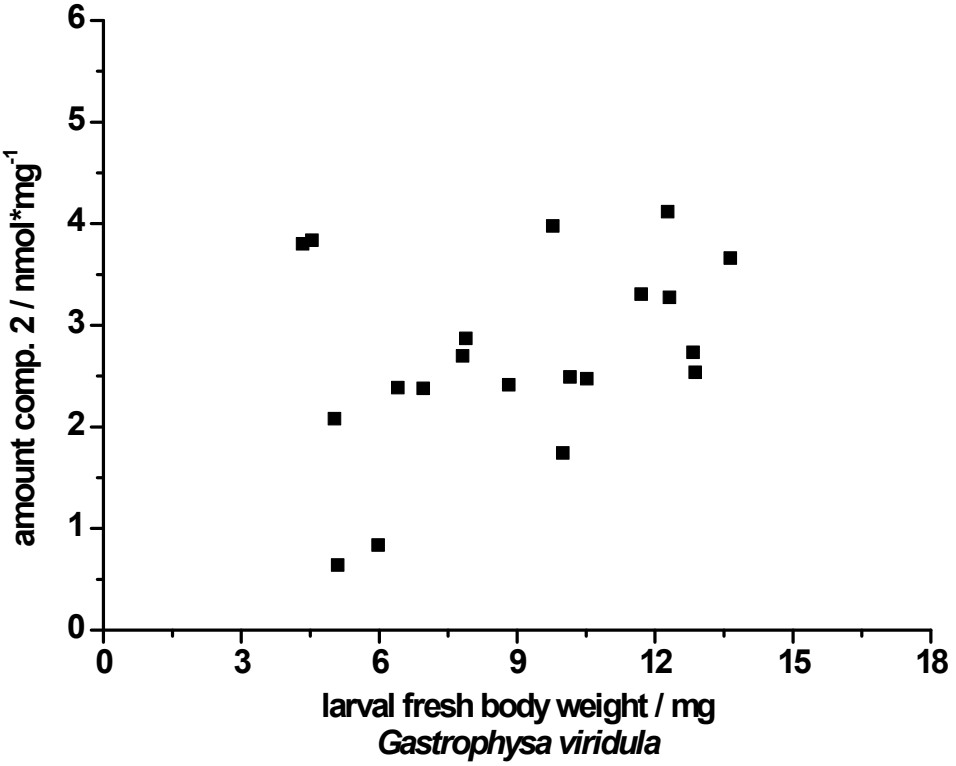


Fig. S11: Molar amount of compound **2** in *Gastrophysa viridula* per body weight, feeding on *Rumex obtusifolius* leafs.

GC-MS measurements of plant extracts

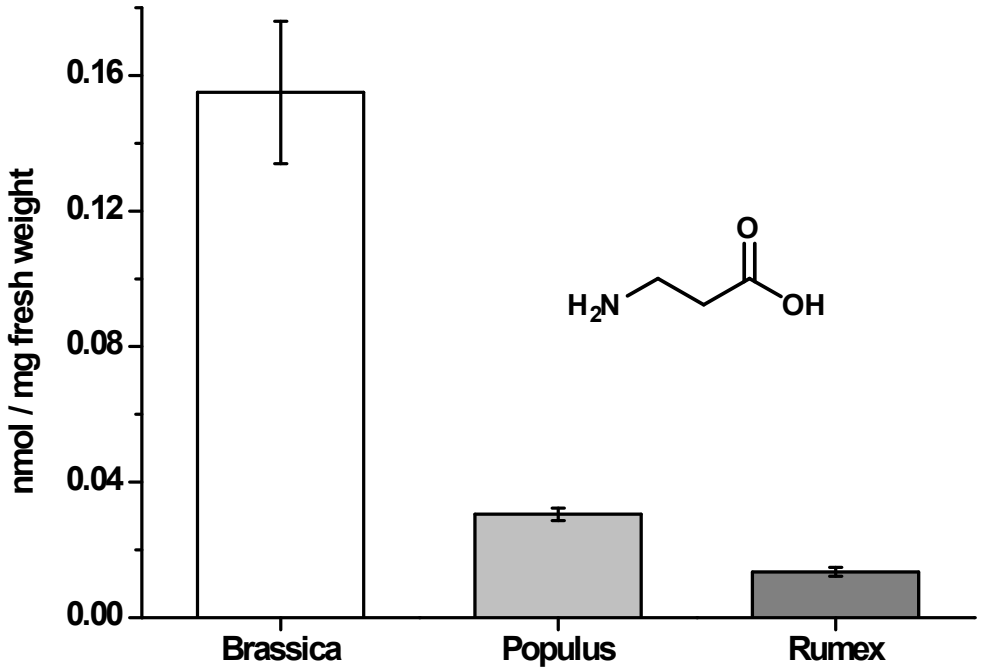


Fig. S12: Molar amounts of free β-alanine in plant leafs per mg fresh weight; n = 7 ± SD.

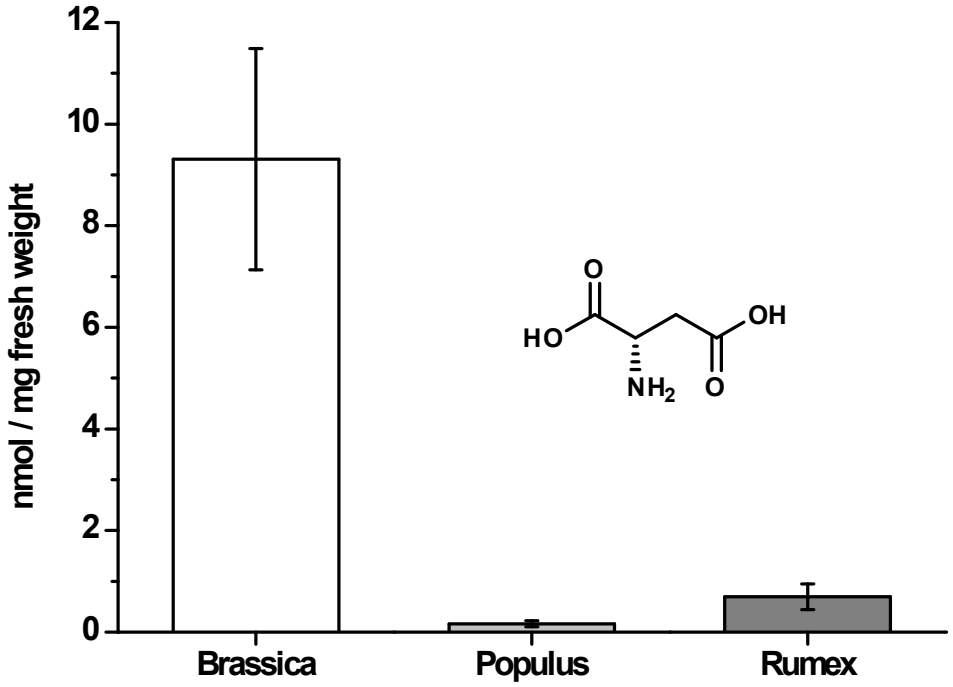


Fig. S13: Molar amounts of free aspartic acid in plant leafs per mg fresh weight; n = 7 ± SD.