3-Nitropropanoic acid and Isoxazolin-5-one derived Glucosides in Chrysomelina Larvae: Synthesis, Biosynthesis, Occurrence and Toxicity

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Preface

“It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of light, it was the season of darkness, it was the spring of hope, it was the winter of despair, we had everything before us, we had nothing before us, we were all going direct to heaven, we were all going direct the other way…“

From: A tale of two cities, written by Charles John Huffam Dickens

Abbreviations

18-cr.-6	18-crown-6 crown ether	**HR**	High resolution
3-NPA	3-nitropropanoic acid	**IR**	Infrared spectroscopy
%	Percent	**I_{rel}**	Relative intensity
°C	Degree celsius	**M**	Molar; mol/l
APCI	Atmospheric pressure chemical ionization	***m*/*z***	Mass-to-charge ratio
		Me	Methyl-
BIA	(β-isoxazolin-5-on-2-yl)-alanine	**MeCN**	Acetonitrile
		MeOH	Methanol
Bn	Benzyl	**MHz**	Megahertz
CALB	*Candida antarctica* lipase B	**µl**	Microliter
$CHCl_3$	Chloroform	**ml**	Milliliter
CoA	Coenzyme A	**mM**	Millimolar; mmol/l
d	Day(s)	**min**	Minutes
DBr	Deuterium bromide	**MS**	Mass spectrometry
DCC	*N*,*N*´-Dicyclohexyl-carbodiimide	***n*-BuLi**	*n*-Butyllithium
		NaOM	Sodium methoxide
DCM	Dichloromethane	**NMR**	Nuclear magnetic resonance
DMAP	*N*,*N*-Dimethylaminopyridine	**PA**	Propynoic acid
DMF	Dimethylformamide	**ppm**	Parts per million
DMSO	Dimethyl sulfoxide	**RNA**	Ribonucleic acid
Equ.	Equivalents	**RNAi**	Ribonucleic acid interference
ESI	Electrospray ionization	**rel.**	relative
Et_2O	(Diethyl) ether	**rt**	Room temperature
EtOAc	Ethyl acetate	**s**	seconds
EtOH	Ethanol	***t*-Boc**	tert.-butyl-oxycarbonyl
Exc.	Excess	***t*-Bu**	tert.-butyl
FA	Formic acid	**THF**	Tetrahydrofuran
GC	Gas chromatography	**TMS**	Trimethylsilyl
h	Hour(s)	**TOF**	Time-of-flight
HCl	Hydrochloric acid	**UDP**	Uridine diphosphate

Figures

1. Introduction

1.1 Biological role of Isoxazolin-5-one and 3-Nitropropanoic acid derivatives

1.1.1. Occurrence

Isoxazolin-5-one and 3-nitropropanoic acid moieties occur in a variety of organisms as structural elements of diverse compounds of biological importance.[1-14] Some examples from this class of natural products are shown below (Fig. 1).

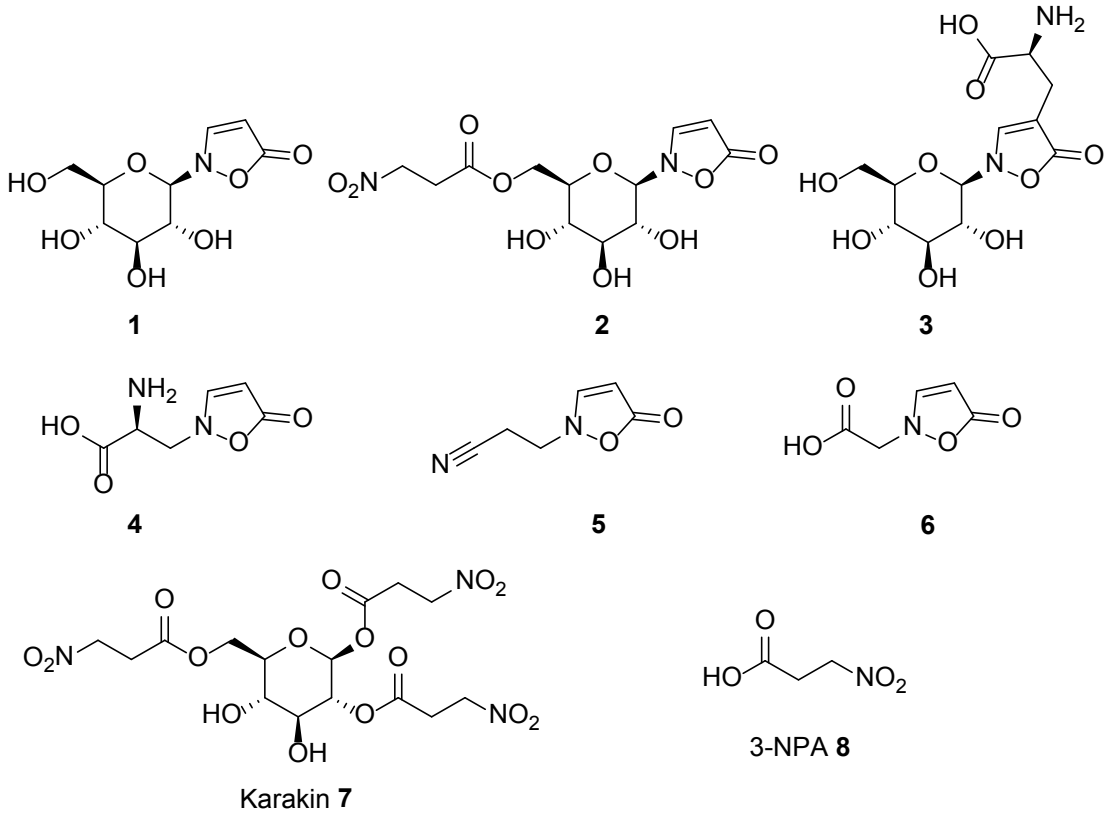


Figure 1 Structures of representative isoxazolin-5-one and 3-NPA derivatives occurring in different organisms (for details see following text and references).[1-14]

The chemical diversity of isoxazolin-5-one and 3-nitropropanoic acid (3-NPA) derived compounds varies from amino acid derivatives of the heterocycle (**3**, **4** and **6**)[4], glucose esters of 3-NPA (**2** and **7**)[5,7,11,12], the free acid itself (**8**)[13,14] or other compound classes, e.g. cyanide derivatives as compound **5**[4].

Adult leaf beetles of the subtribe Chrysomelina (Chrysomelinae: Coleoptera), e.g. *Chrysomela populi* (Fig. 2), contain isoxazolin-5-one and 3-NPA derived glucosides **1** and **2** as major components in their elytral secretions as well as in their eggs.[5,9,15]

Figure 2 Adult *Chrysomela populi* on *Populus* sp.; compounds **1** and **2** occur in the elytral secretions.

As isoxazolin-5-one derivatives **1** and **2** occur only in leaf beetle species of the subtribe Chrysomelina, they were identified as taxonomic characters for the classification of these insects into this subtribe.[16,17]

In contrast to the adult beetles, the larval defensive secretions contain volatiles that derive from sequestered compounds, provided by the beetle´s host plant or by *de novo* production of corresponding precursors.[18-27] These volatiles, e.g. salicyl aldehyde in juvenile *Chrysomela populi*, have been shown to deter predators and exhibit toxicity to insects as well.[28]

In the host plants of leaf beetle species belonging to the Chrysomelina subtribe, e.g. *Populus* spp. (host plant family of e.g. *C. populi*) or *Rumex* spp. (host plant family of e.g. *Gastrophysa viridula*), neither isoxazolin-5-one nor 3-NPA derivatives were detected so far. This indicates possible *de novo* production of such moieties by the insects. In contrast, all shown derivatives **1-8** occur in many other plant species, mainly belonging to the legume family (Fabaceae) as well as to the Malpighiaceae, Corynocarpaceae and Violaceae.[3,8,11,14,29-33] In case of Fabaceae, a total number of 19500 species within approximately 751 genera was estimated, representing one of the biggest plant families.

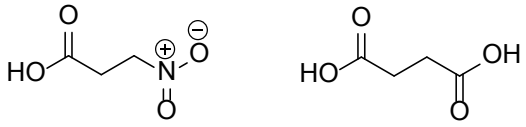
Thus, the potential of the ecological importance of 3-NPA and isoxazolin-5-one derivatives is indicated, which might significantly contribute to the tremendous evolutionary success of this plant family, besides other factors (e.g. nitrogen fixation).[14,34,35]

The free 3-nitropropanoic acid **8** is produced by certain fungi species as well, belonging to *Arthrinium*, *Aspergillus*, and *Penicillium* tribes.[11,32,36-40] Early publications describe the occurrence of 3-NPA in *Aspergillus flavus*.[41] The demonstrated widespread occurrence of isoxazolin-5-one and 3-NPA motivs in compounds present in organisms belonging to different kingdoms and diverse families directly leads to the question of ecological benefits for their producers. The following chapter will address parts of this aspect, describing phenomena of toxicity and detoxification of isoxazolin-5-one and 3-NPA derived compounds that have been reported prior to this thesis.

1.1.2. Toxicology

Toxicity of 3-nitropropanoic acid

3-Nitropropanoic acid **8** is isoelectronic to succinic acid (Fig. 3), which is a key intermediate in the citric acid cycle.



3-nitropropanoic acid **8** (3-NPA) succinic acid

Figure 3 Structures of inhibitor and actual substrate of succinate dehydrogenase.

Due to this property, both compounds are able to bind to the catalytically active center of succinate dehydrogenase. The latter enzyme catalyzes the transformation between succinate and fumarate, one step within the citric acid cycle.[42] Consequently, it has been observed that 3-NPA **8** inhibits succinate dehydrogenase.[43] Furthermore, it was demonstrated that the inhibition is irreversible, due to a covalent interaction between 3-NPA **8** and the catalytic center of succinate dehydrogenase, as shown by x-ray diffraction.[44,45] Thus, the ATP-generation upon oxidation of succinate to fumarate is

disturbed.[42] These toxic effects of 3-NPA **8** affect especially nerve cells.[46,47] *In vivo* studies with ants (*Myrmica rubra*) feeding on sucrose solutions of compounds **1** and **2** (compare section 1.1.1.) showed increased cumulative mortality in case of the 3-NPA-ester **2**.[15] In addition, the latter compound showed deterrent effects in binary choice tests with these ants.[15] No effect in terms of mortality and deterrence was observed in case of non 3-NPA-derived compound **1**.[15] The isoxazolin-5-one and 3-NPA-derived compounds **1** and **2** were applied in 10^{-1} to 10^{-2} M concentrations, representing (hyper-)physiological amounts of these substances in the defensive secretions of the adult leaf beetles.[15] Similar experiments have been carried out with additional 3-NPA derived glucosides, isolated from the adult secretions of Japanese leaf beetles (Chrysomelina).[9] 3-NPA-esters of glucose, lacking the isoxazolin-5-one moiety, e.g. Karakin **7** (compare section 1.1.1.), isolated from legumes, have been shown to be toxic against Grass Grub (*Costelytra zealandica*; Coleoptera: Scarabaeidae).[48]

Detoxification of 3-nitropropanoic acid

Although the toxicity of 3-NPA **8** has been demonstrated unambiguously, several organisms developed different detoxification pathways.[11,49-51] Due to feeding on plants producing 3-NPA **8** and its derivatives, a significant resistance against 3-NPA **8** toxins evolved in ruminants.[11] It was shown that the microbiome from the gut of cow and sheep are able to metabolize 3-NPA **8** to produce intermediate β-alanine, which is further metabolized by the microorganisms (Fig. 4).[11,49,52]

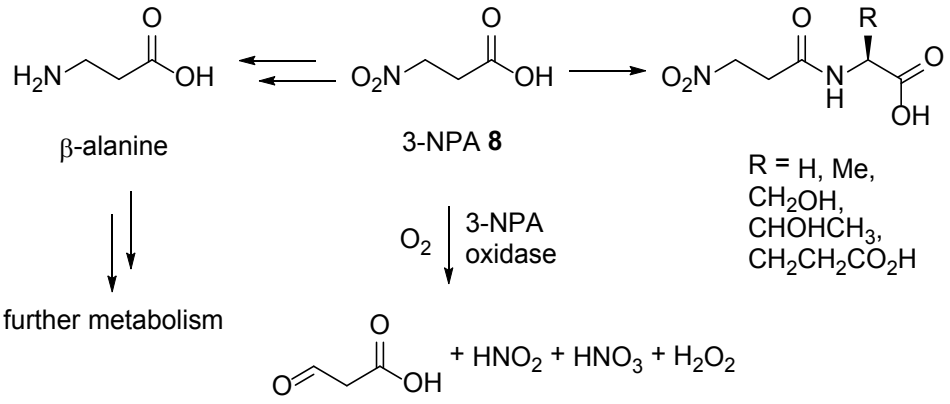


Figure 4 Detoxification products of 3-NPA in some microorganisms, plants and insects.[13,14,50-52]

An alternative pathway for detoxification of 3-nitropropanoic acid **8** was found in case of two grasshopper species *Melanoplus bivittatus* and *Melanoplus sanguinipes* (Caelifera: Melanoplinae) as well as the cotton leafworm *Spodoptera littoralis* (Lepidoptera).[50,51] In both cases amides of 3-NPA and proteinogenic amino acids were detected as detoxification products (Fig. 4).[50,51] After uptake over the gut and conjugation with amino acids, the detoxification products are excreted via the frass of the insect.[51] In case of *S. littoralis* evidence for the detoxification by the insect itself was provided, rather than a participation of its intestinal microbiome.[51]

A third detoxification pathway was discovered in plants belonging to the legume family that produce free 3-NPA.[13,14] In case of *Hippocrepis comosa* and other fabaceous plants 3-NPA is oxidized by the enzyme 3-NPA oxidase under consumption of oxygen, forming malonate semialdehyde associated with nitrate, nitrite as well as hydrogen peroxide (Fig. 4). Due to migration of the toxin from the shoots into the phloem, this detoxification within 3-NPA producers is supposed to be a protection mechanism for the plant to maintain mitochondrial activity.[13]

Toxicity of Isoxazolin-5-one derivatives

Some of the above shown isoxazolinone derivatives (see section 1.1.1.), e.g. (β-isoxazolin-5-on-2-yl)-alanine BIA **4**, occurring in legumes, show neurotoxic effects that are linked to the disease of lathyrism.[53,54] *In vitro* assays with *Lathyrus sativus* samples provided evidence for the role of BIA **4** as a precursor for L-2,3-diaminopropanoate (DAP) and β-*N*-oxalyl-L-2,3-diaminopropanoic acid β-ODAP (Fig. 5).[8,55]

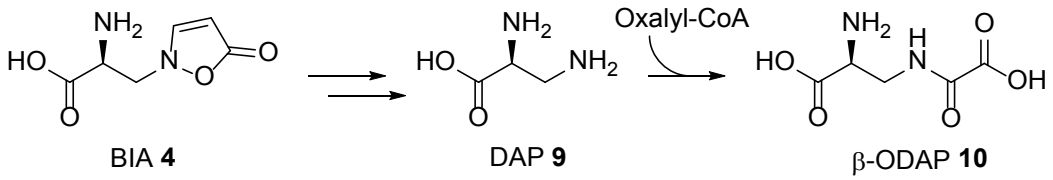


Figure 5 Transformation of BIA **4** into the toxic compounds DAP **9** and β-ODAP **10**.[56]

β-ODAP **10** occurs in significant amounts in *Lathyrus* species and is assumed to be mainly responsible to cause neurolathyrism in higher animals upon consumption of

excessive amounts of e.g. *Lathyrus sativus* seeds.[56,57] In contrast, DAP **9** is described as an intermediate in *Lathyrus sativus*, which is directly transformed into β-ODAP.[56] Nevertheless, it has been shown that DAP **9** is a growth inhibitor for some bacterial strains, due to inhibition of proline, pantothenate and isoleucine biosynthesis.[58,59]

Furthermore, a number of non-biologically occurring 2- and 3-substituted isoxazolin-5-one derivatives showed antifungal activity (Fig. 6).[60]

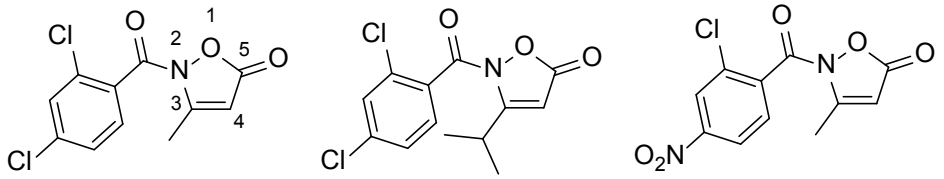


Figure 6 3-Isoxazolin-5-one derivatives **11**, **12** and **13** with antifungal activity.[60]

The above mentioned compounds were tested against plant pathogens, e.g. *Phythophthora infestans*, *Septorii apii* and *Alternia solari conida*, showing altering general fungicidal activity and mycelial inhibition.[60]

However, no direct toxic or repellent effects of the similar isoxazolin-5-one glucoside **1** (compare section 1.1.1.) have yet been demonstrated.[15]

1.1.3. Biosynthesis

3-NPA moiety

In case of *Penicillium atrovenetum* the biosynthetic pathway was investigated by different groups in several previous studies.[32,36-40] In the mentioned fungus, it was demonstrated that the biosynthesis of 3-nitropropanoic acid **8** derives from aspartate (Fig. 7, top). In several subsequent steps, aspartate is oxidized at the nitrogen atom, leading to the formation of intermediate (*S*)-nitrosuccinate **14**.[38] The oxygen atoms for the oxidation steps derive from dioxygen, which is consumed via monooxygenation of the *N*-position.[37] Upon decarboxylation of compound **14**, 3-NPA **8** is formed in a final step. Derivatives of β-alanine failed to be incorporated in the biosynthetic steps of 3-NPA, which provides evidence that the suggested pathway is the most important source for this compound in *P. atrovenetum*.[40]

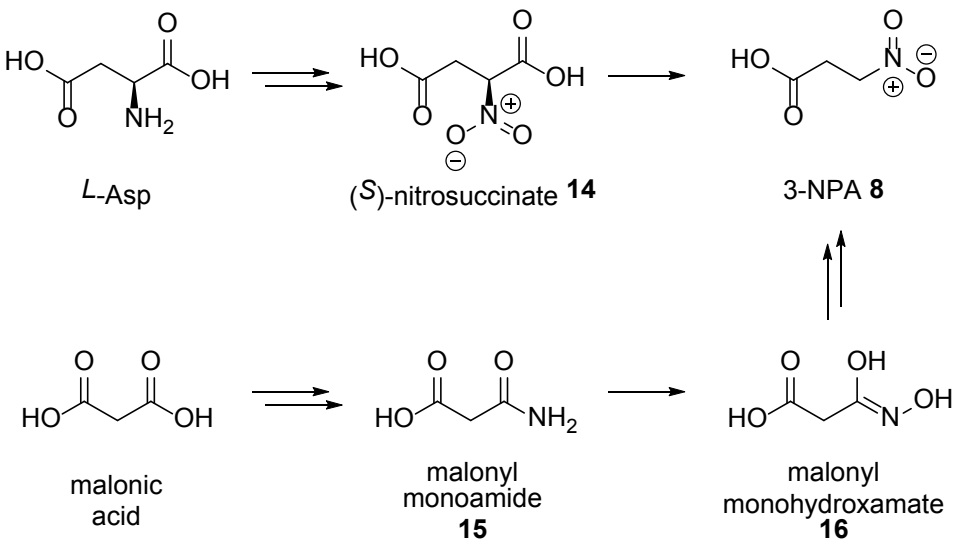


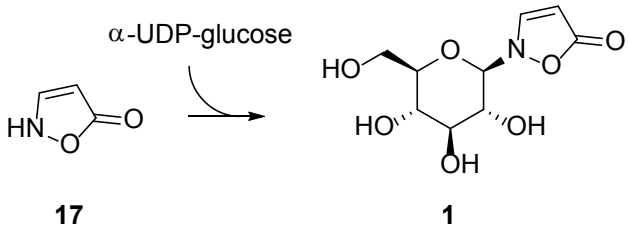
Figure 7 Proposed biosynthetic pathways for the formation of 3-NPA in *Penicillium atrovenetum*, adapted from Baxter *et al.*, top[37] and in *Indigofera spicata*, adapted from Candlish *et al.*, bottom.[61]

These findings have been adapted to investigate the metabolic route of the 3-NPA production in adult leaf beetles of the species *Chrysomela tremulae*.[6] For this purpose, solutions of [$^{14}C_4$]-aspartate were applied to leaves of the food plant of *C. tremulae* and presented to the adults. After one week, radioactivity could be detected in compounds **1** and **2** that were isolated from the defensive secretions. This result indicated that compounds **1** and **2** can be *de novo* produced by the adult beetles, starting from aspartate. However, further intermediates as well as intactness of incorporation for the suggested pathway have not been characterized to support this hypothesis. Studies on the biosynthesis of 3-nitropropanoic acid in creeping indigo (*Indigofera spicata*) indicated a different pathway, deriving from malonate as a starting compound (Fig. 7, bottom).[61] Malonate is proposed to be further transformed into malonyl monoamide **15** in this sequence. Via formation of intermediate malonyl monohydroxyamate **16**, 3-NPA might be formed in *I. spicata*. These suggestions are evidenced by application of [2-^{14}C]-malonate as well as [2-^{14}C]-malonyl monohydroxyamate to the plants *in vivo* or *in vitro*.[61]

Isoxazolin-5-one moiety

One of the few results that have been published prior to this work about the formation of the isoxazolin-5-one heterocycle refer to the already mentioned publication by Pasteels and coworkers (see section 1.1.3., 3-NPA moiety).[6] In this study, feeding of [$^{14}C_4$]-aspartate evidenced the formation of the heterocycle by *de novo* biosynthesis in adult *Chrysomela tremulae*, without providing information in terms of further intermediates on the pathway. Furthermore, a first indication for a link between the formation of the 3-NPA moiety and the heterocycle is provided by these findings.

In plants, only the derivatization of the heterocycle **17** has been investigated so far.[62-64] It was demonstrated that BIA **4** derives from *O*-acetyl-serine, while compound **1** derives from α-UDP-glucose (Fig. 8).[63]



17 **1**

Figure 8 Proposed final step in the biosynthesis of isoxazolin-5-one glucoside **1** in *Pisum* and *Lathyrus* seedling.[63]

Compound **17** was considered as a substrate in any of these studies, but its formation was not described prior to this work in any detail, neither in plants, nor in leaf beetles. To address further questions concerning the biosynthesis of isoxazolin-5-one and 3-NPA derived glucosides, the chemical synthesis of such derivatives is of interest. In the following chapters useful previously described synthetic strategies for the construction of such compounds are discussed.

1.2. Synthesis and properties of Isoxazolin-5-one and 3-NPA derived glucosides

1.2.1. Acylation of glucosides

For the synthesis of glucose esters, e.g. compound **2** (see section 1.1.1.), a number of synthetic methods has been developed. The challenge of these transformations refers to

the selective derivatization of the hydroxyl functions. For this purpose, the use of selective protection and deprotection of suitable precursors is a valid strategy (Fig. 9).[65]

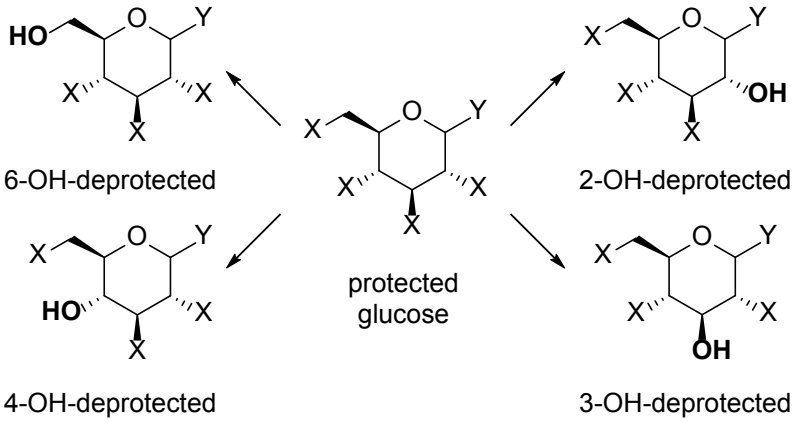


Figure 9 Principle of selective deprotection of glucose precursors; X = TMS, Y = α-OMe; for details see Wang *et al*.[65]

The shown protected starting agent is commercially available and the selectively deprotected product can be further transformed by acylation reactions, e.g. carbodiimide couplings.[66] Since it is of importance in following chapters of this thesis, the mechanism of such carbodiimide acylations is shown below (Fig. 10).[67]

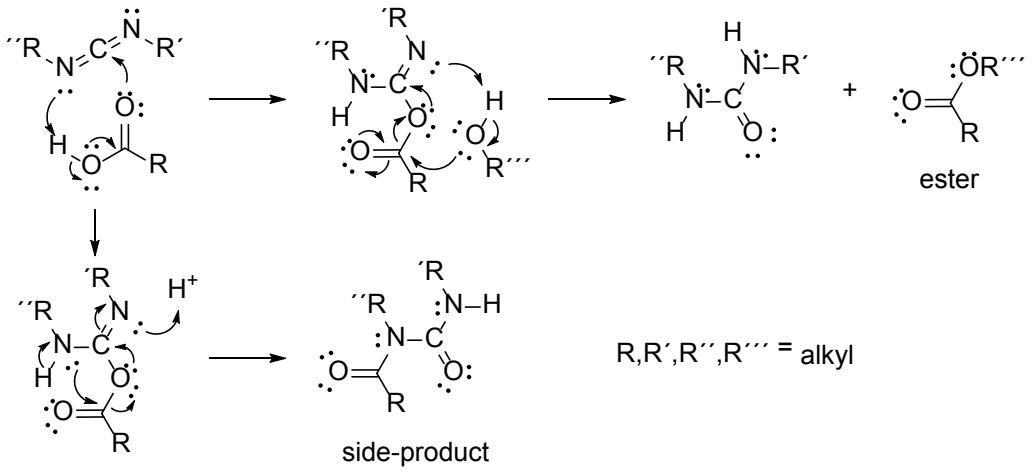


Figure 10 One of the proposed mechanisms of carbodiimide coupled esterifications.[67]

An alternative method for selective acylation is the use of catalysts, when starting from partial or completely unprotected precursors. These catalysts can be based on naturally occurring enzymes[68-71] or synthetic products[72] as well. In many cases, commercially

available immobilized *Candida antarctica* lipase B (CALB) is used to transform unprotected glucosides into the corresponding 6-*O*-ester (Fig. 11).[70,71]

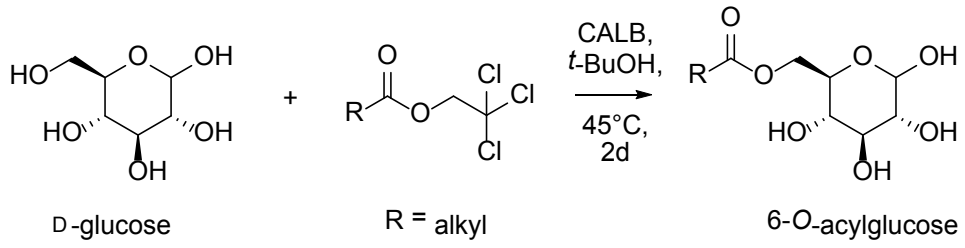


Figure 11 Transesterification of an acyl-donor and D-glucose using CALB as a catalyst.[70,71]

The advantages of strategies based on enzymatic catalysis lie in the reduction of the number of synthetic steps. In addition, expensive protected starting material can be circumvented in many cases. On the other hand, the yields of such reactions are not quantitative in many cases, depending on the acyl-donor, among other factors. Due to the mild reaction conditions, most of the reactants remain non-transformed, allowing a recovery of starting material. Furthermore, different functional groups are tolerated.

1.2.2. Isoxazolin-5-one and glycosylation

The synthesis of the isoxazolin-5-one heterocycle **17** has first been described by De Sarlo and coworkers.[73] In this study, compound **17** was synthesized from ethyl propynoate in medium yields (Fig. 12).

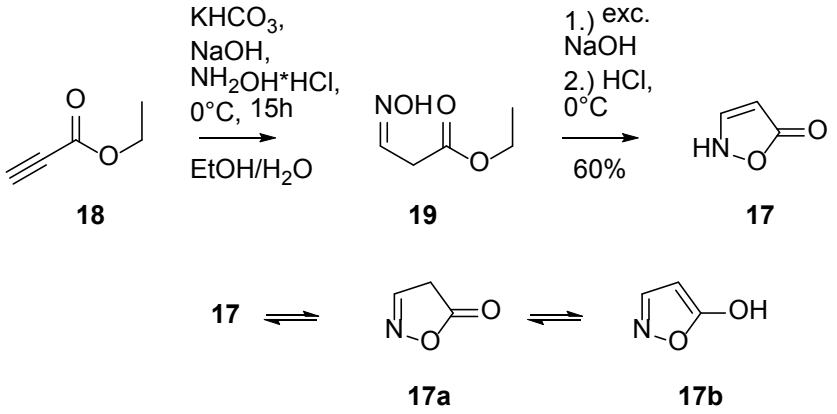


Figure 12 Synthesis of isoxazolin-5-one **17** and its tautomers **17a** and **17b** respectively; the total yield is given.[73]

The content of the single tautomers **17**, **17a** and **17b** in the equilibrium depends on the polarity of the solvent.[73] Having access to compound **17**, van Rompuy *et al*. described the syntheses of naturally occurring derivatives of isoxazolin-5-one by substitution reactions.[2] Due to the mentioned tautomerism as well as the low reactivity of the heterocycle, the overall yields of these reactions were small. The substitutive Koenigs-Knorr approach for the synthesis of glucoside **1** resulted in a total yield of only 1 % (Fig. 13).[3,4]

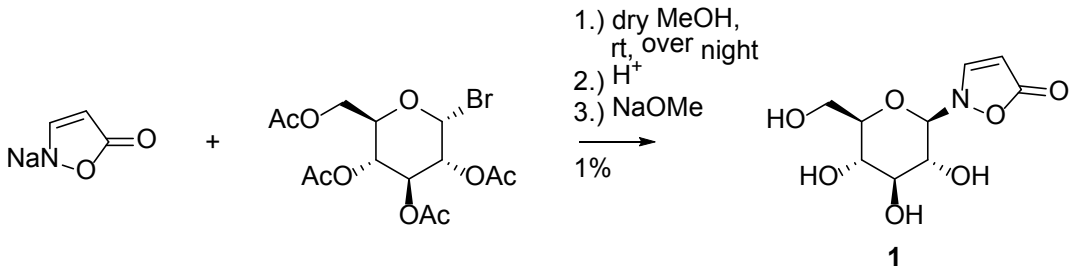


Figure 13 Synthetic protocol for the synthesis of compound **1** by substitution of α-acetobromoglucose with the anion of isoxazolin-5-one.[3,4]

The described low yields reached in this strategy are due to alternative substitution as well as the instability of compound **1** under high pH conditions, as discussed in section 2.1.

Isoxazolin-5-one derivatives, e.g. compound **1** and other biologically occurring products, were characterized in terms of their photochemical properties as well as stability under different pH conditions. It was observed that these derivatives possess high photochemical activity upon irradiation into their absorption maxima around 260 nm. Under these conditions, the quantum yields for the photodegradation in water were determined to be in a range of 0.34 to 0.62.[4] Glucose and glutamic acid were identified as degradation products of compound **3** in such experiments.[4] In addition, it was described that under alkaline conditions the *N*-substituted isoxazolin-5-ones decompose,[4] while the free heterocycle itself shows higher stability in alkaline aqueous media and degrades rapidly under acidic conditions.[73]

Due to the generally occurring low yields that were achieved using the substitution strategy, Baldwin and coworkers developed an alternative method to synthesize isoxazolin-5-one systems based on a 5-endo-dig reaction (Fig. 14).[74,75]

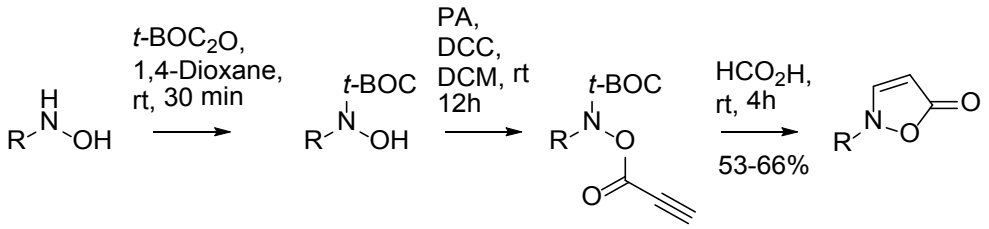


Figure 14 Synthetic strategy for the synthesis of *N*-substituted 3,4-unsubstituted isoxazolin-5-one derivatives based on a 5-endo-dig reaction; R = *t*-Bu, cyclopentyl, Me and Bn; total yields are given.[74,75]

The above mentioned 5-endo-dig strategy was applied to synthesize aliphatic *N*-substituted derivatives of isoxazolin-5-one to yield naturally occurring amino acid as well as other derivatives of the heterocycle.[75,76] However, the 5-endo-dig strategy has not yet been applied for the synthesis of isoxazolin-5-one glycosides, e.g. compounds **1** or **2**. This alternative approach for the synthesis of compounds **1**, **2** and other glycosides is discussed in section 2.1.

1.2.3. 3-Nitropropanoic acid and propanoate derivatives

For studies concerning the biosynthesis of the 3-nitropropanoic acid derivatives, the syntheses of stable-isotope-labeled 3-NPA and other useful products have previously been described.[37,76] The synthesis of [1-^{13}C, 2,2-D_2]-3-nitropropanoic acid was achieved by a three-step synthetic route starting from Na^{13}CN and 2-chloroethanol. The deuterium atoms were introduced by DBr/D_2O. In a final step, the nitro group was constructed using $NaNO_2$ as a substituting agent (Fig. 15).[37]

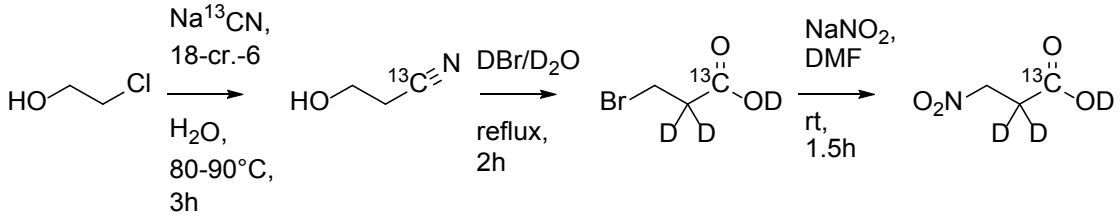


Figure 15 Synthesis of 3-[1-^{13}C,2,2-D_2]-nitropropanoic acid, described by Baxter *et al*.[37]

^{13}C-labeled propynoic acid, as a useful precursor of isoxazolin-5-one and 3-NPA derivatives, has been synthesized by Baldwin and coworkers (Fig. 16).[76]

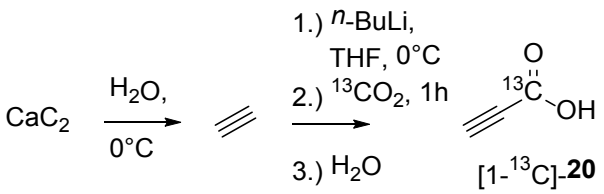


Figure 16 Synthesis of [1-^{13}C]-propynoic acid **20**, described by Baldwin and coworkers.[76]

The above described synthetic methods were used to synthesize intermediates to unravel the biosynthesis of isoxazolin-5-one and 3-NPA derivatives in Chrysomelina larvae (see section 2.3.1.).

1.3. Aims of this thesis

In the previous sections it was shown that isoxazolin-5-one and 3-NPA derivatives **1** and **2** occur in the defensive secretion of adults and the eggs of leaf beetles, belonging to the subtribe Chrysomelina. Some aspects of the biosynthesis of these compounds have been addressed by feeding of ^{14}C-labeled aspartic acid to *Chrysomela tremulae* adults. Compound **1** has already been synthesized by a substitution strategy, providing very poor yields of around 1 %. In addition, no biological function could be shown in case of compound **1**. In order to study further details in terms of the occurrence, biosynthesis, chemical synthesis and biological functions of compounds **1** and **2** in Chrysomelina, alternative synthetic routes for these molecules were of interest. The synthesized compounds should then be used as authentic (stable-isotope-labeled and non-labeled) standards for identification and quantification of the biological occurring products. The toxicity of compound **1** against cell cultures was of interest. The (physico-)chemical properties of isoxazolin-5-one glycosides should be addressed, on the basis of the synthetic standards. It was of interest to study all life stages and compartments of these insects by NMR- and LC-MS-techniques as well. Furthermore, it was of interest to unravel the biosynthetic pathway of compounds **1** and **2** using *in-vivo*- and *in-vitro*-application of commercial and synthetic stable-isotope-labeled and non-labeled putative intermediates.

2. General Discussion

2.1. Synthesis and properties of isoxazolinone and 3-NPA derivatives

2.1.1. Synthesis

For the synthesis of isoxazolin-5-one derived glucosides, the 5-endo-dig strategy, developed by Baldwin and coworkers[75], was applied (Fig. 17).[77]

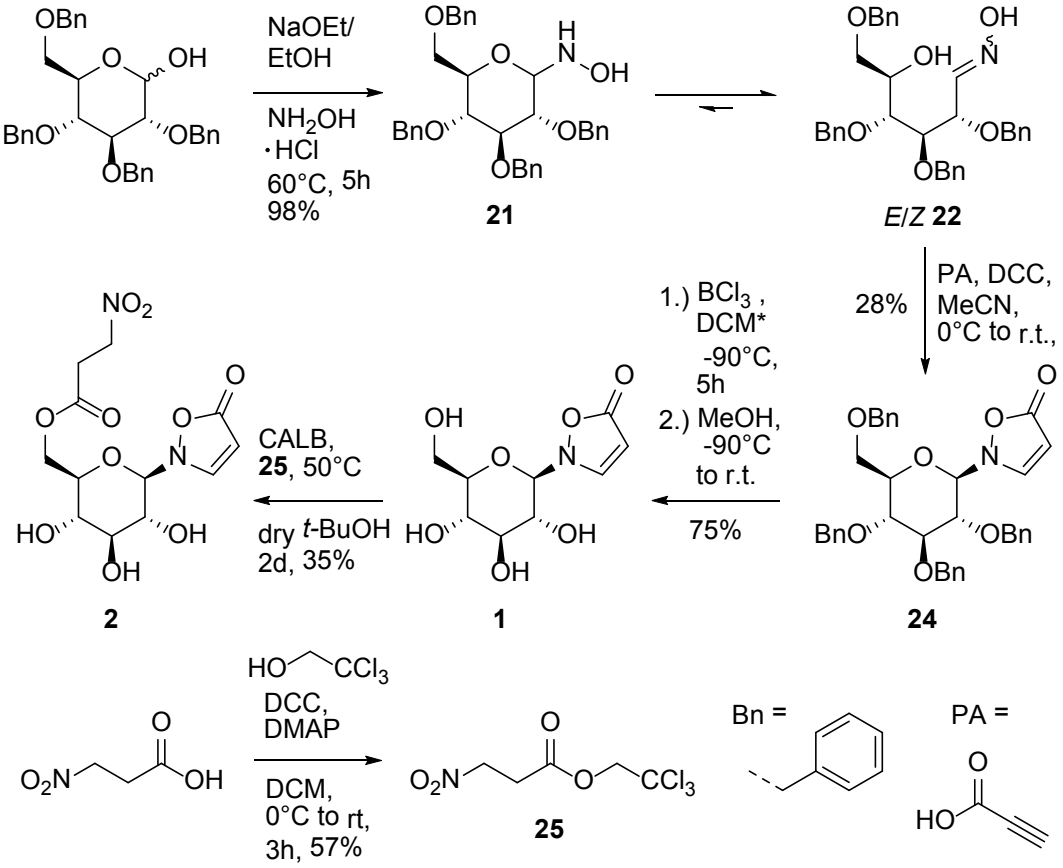


Figure 17 Synthesis of compounds **1** and **2**, based on a 6-exo-trig-5-endo-dig cascade reaction, followed by deprotection and regioselective transesterification.[77]

For this purpose, a tetra-*O*-benzyl protected *N*-Boc-*N*-hydroxyglucopyranosylamine was a rational intermediate that should be synthesized in two steps from tetra-*O*-benzylglucose. However, the cyclic pyranose compound **21** is only the minor isomer, while predominantly the non-cyclic *E*- and *Z*-oximes **22** are formed.[78] ^{1}H NMR measurements showed that the equilibrium between the open-chain oximes and the cyclic

pyranose isomer can be influenced by the applied solvent. In case of acetonitrile only the open chain form was observed, while in chloroform the ratio between oxime **22** and pyranose **21** was about 3 to 1 (Fig. 17).[77] Due to these circumstances, an effective *N*-Boc-protection/deprotection of *N*-hydroxyglucopyranosylamine **21** failed. Alternatively, the oxime forms *E*- and *Z*-**22** were directly converted with DCC-activated propynoic acid. As a result, the α and β-isomers of the desired corresponding tetra-*O*-benzyl-protected isoxazolin-5-one glucosides **23** and **24** were obtained in moderate yields (35%). To circumvent an acylation at the C(5)-OH group, the DCC coupling was carried out without the use of DMAP as a catalyst, exploiting the intrinsic differences in the reactivity as well as the steric hindrance of the secondary C(5)-OH compared to the primary N-OH group. Additionally, the reactants were slowly mixed over 20 min to prevent from double acylation (at the NOH- and C-5´-OH-position).[77] ^{1}H NMR measurements show that the acylation at the NOH group is nearly quantitative. The long reaction time (7d) provides evidence for a high energy barrier, probably due to the formation of the 5-membered isoxazolin-5-one ring. However, higher temperatures after the acylation could not improve the yield significantly.

The mechanism of the reaction leading to the isoxazolin-5-one products **23** and **24** is supposed to proceed via a 6-exo-trig-5-endo-dig cascade (Fig. 18).[77]

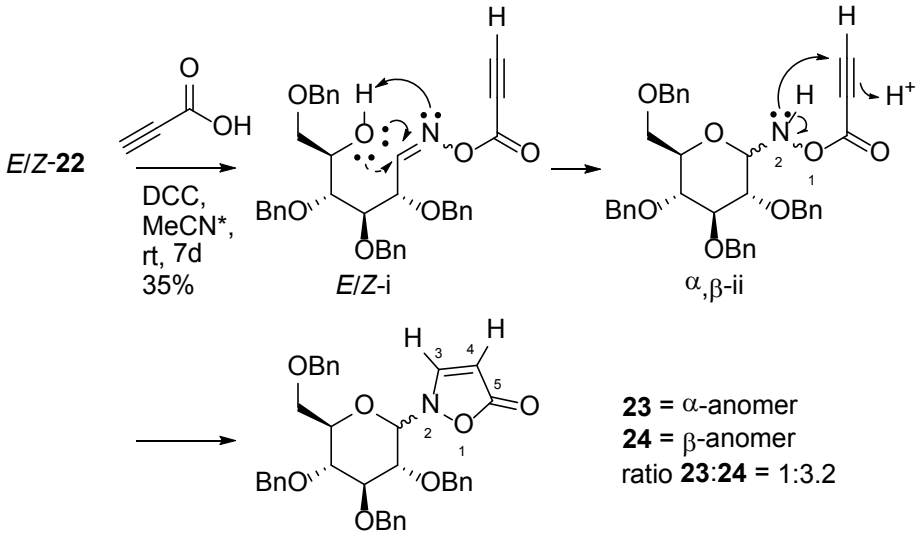


Figure 18 Proposed mechanism for the cascade reaction.[77]

Online NMR measurements of the reaction mixture provided evidence for this hypothesis.[77] The purification of compounds **23** and **24** was successful using preparative low-pressure column chromatography on two consecutive silica columns, eluted with DCM/MeCN 100:1 and $CHCl_3$/EtOAc 95:5. The β-isomer could be crystallized from EtOAc and an x-ray crystal structure analysis of this sample was performed (Fig. 19).[77]

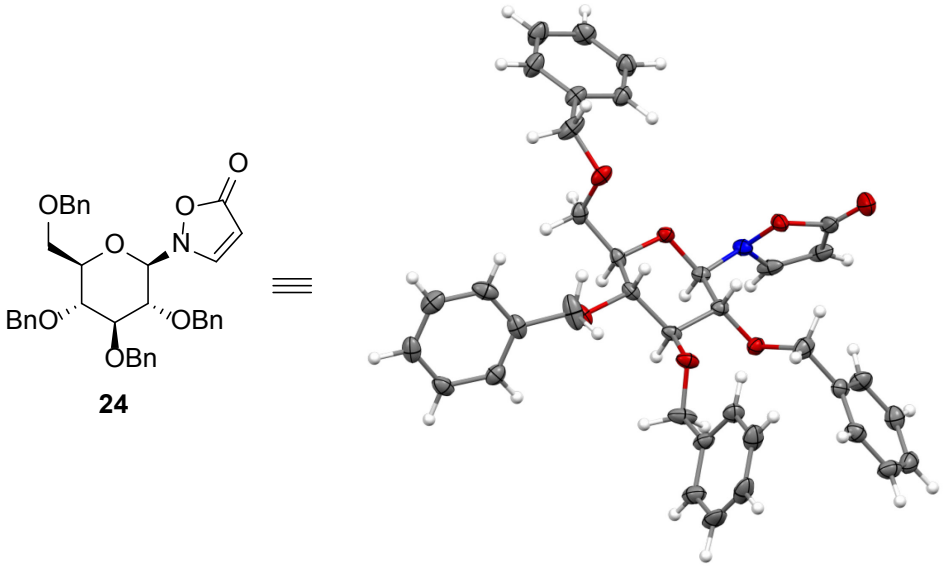


Figure 19 X-ray structure of compound **24**, showing ellipsoids with 50% probability; Grey = carbon, white = hydrogen, red = oxygen, blue = nitrogen.[77]

The above shown crystal structure of compound **24** is equivalent with a structure alignment of the heterocycle as a 3-isoxazolin-5-one ring.

To unprotect the tetra-*O*-benzyl derivatives, a reductive strategy was not applied due to the α,β-unsaturated carbonyl moiety. Instead, BCl_3 was used as a reagent at low temperatures in ether, yielding the unprotected target molecule **1**.[77,79] Oxidative attempts using DDQ for the deprotection of **24** yielded complex mixtures of the incompletely deprotected precursor. The overall yield of compound **1** in the novel described sequence is around 22%, which is a significant improvement of the earlier described route that yielded only 1% of the compound.[4,77] Compound **2** could be synthesized by using the trichloro-activated ester of 3-NPA **25**, which reacted with compound **1** under regioselective transesterification using commercial immobilized *Candida antarctica* lipase B (CALB). The yield of the latter reaction was in the range that is described for

such transformations[70] (around 35%) and the starting material (compound **1**) can be particularly recovered within the column chromatography purification (Silica, EtOAc/MeOH/DCM 10:1:1 to 2:1:0).[77] The activated ester **25** was synthesized using commercial 3-NPA and 2,2,2-trichloroethanol under Steglich-conditions.[66]

In order to access further isoxazolin-5-one derived glycosides, the synthetic route for the construction of the heterocycle was further simplified by the use of unprotected sugars as starting reagents. The novel protocol was applied to D-glucose, D-fructose, D-maltose, D-xylose, D-ribose as well as D-2-deoxyribose (Fig. 20).[80]

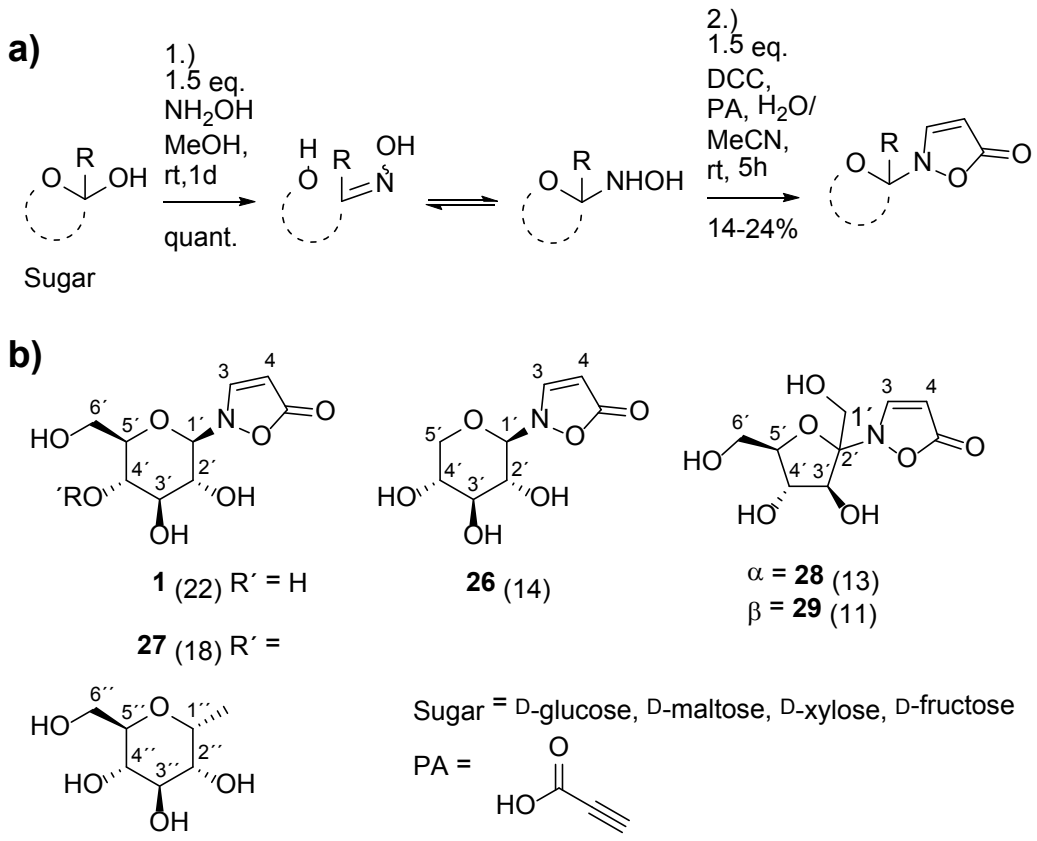


Figure 20 Synthetic protocol (a) and structures (b) for the synthesis of isoxazolin-5-one glycosides applying the one-pot protocol; yields are given in brackets; quant. = quantitative.[80]

This method for the synthesis of isoxazolin-5-one glycosides was successful in case of glucose, maltose, xylose and fructose and failed using ribose and 2-deoxyribose as starting material. The isolated yields for the successfully synthesized single compounds were in the range of 11 to 22%. The number of steps starting from commercial material

was reduced from three to two and the reactions were carried out in one pot. For the purification column chromatography based on MeCN/H_2O mixtures on silica were used, yielding pure material that crystallized reproducibly in case of glucose and maltose. For the naturally occurring glucose derivative **1** a crystal structure could be obtained by single crystal x-ray diffraction, again performed and kindly provided by Dr. Helmar Görls (Fig. 21).

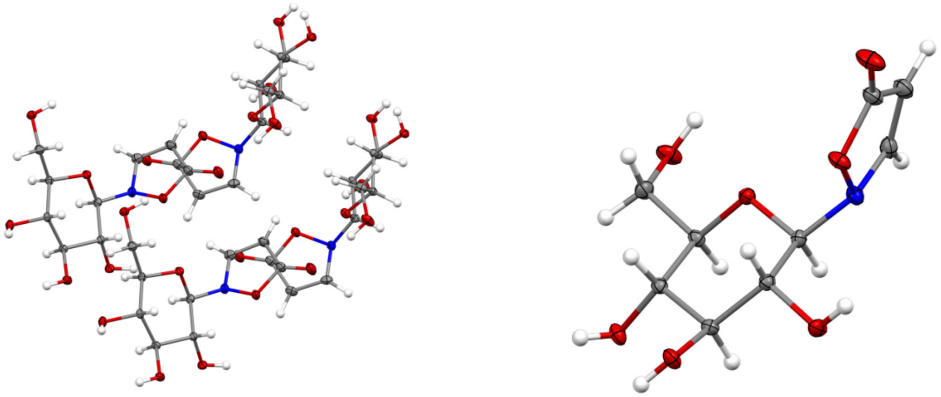


Figure 21 Unpublished x-ray structure of compound **1**, showing ellipsoids with 50% probability; Grey = carbon, white = hydrogen, red = oxygen, blue = nitrogen; left: packing, right: single molecular structure.

The formation of compounds **1**, **26**, **27**, **28** and **29** derives from easily accessible *N*-hydroxyamino glycosides that were synthesized in the first step in quantitative yields, according to literature procedures.[81] These intermediates exhibit an equilibrium between the open-chain *E*/*Z*-oxime forms and the corresponding cyclic *N*-hydroxy derivatives. In case of solutions of the reducing sugars (glucose, xylose, maltose and ribose) the oxime forms mainly predominate, while the ring forms are the only isomers present in the crystalline precipitate.[81] In case of fructose the open chain forms are the only isomers that can be observed in both, solutions as well as precipitates. Due to these observations the proposed mechanisms may succeed via a 5-endo-dig pathway (in case the ring form is the starting material) or a 6-exo-trig-5-endo-dig cascade (in case of open chain oximes, Fig. 22).[80]

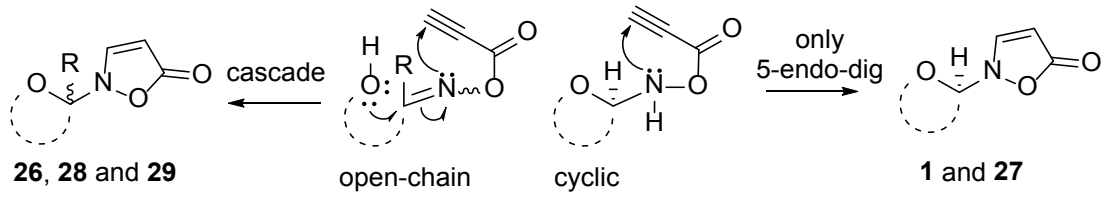


Figure 22 Proposed mechanisms of the formation of products **1** and **26** to **29**.[80]

The structure assignment was carried out using 1D and 2D NMR experiments (for details see the literature data[77,80]) being supported in case of glucose by x-ray crystal structure analyses. In case of fructose (lacking an anomeric proton), the structure was assigned by comparison of the NMR and other data with literature results of similar fructofuranosides and -pyranosides.[82-84]

It is supposed that the observed variations in terms of the yields and α/β-selectivities for the products depend on the nature of the starting material (open chain vs. cyclic isomers). This hypothesis is evidenced by the comparison of the α/β-selectivities between the above mentioned protecting group strategy based synthesis vs. the protecting group free route. The selectivities in both cases were determined via ^{1}H NMR measurements (Fig. 23).[77,80]

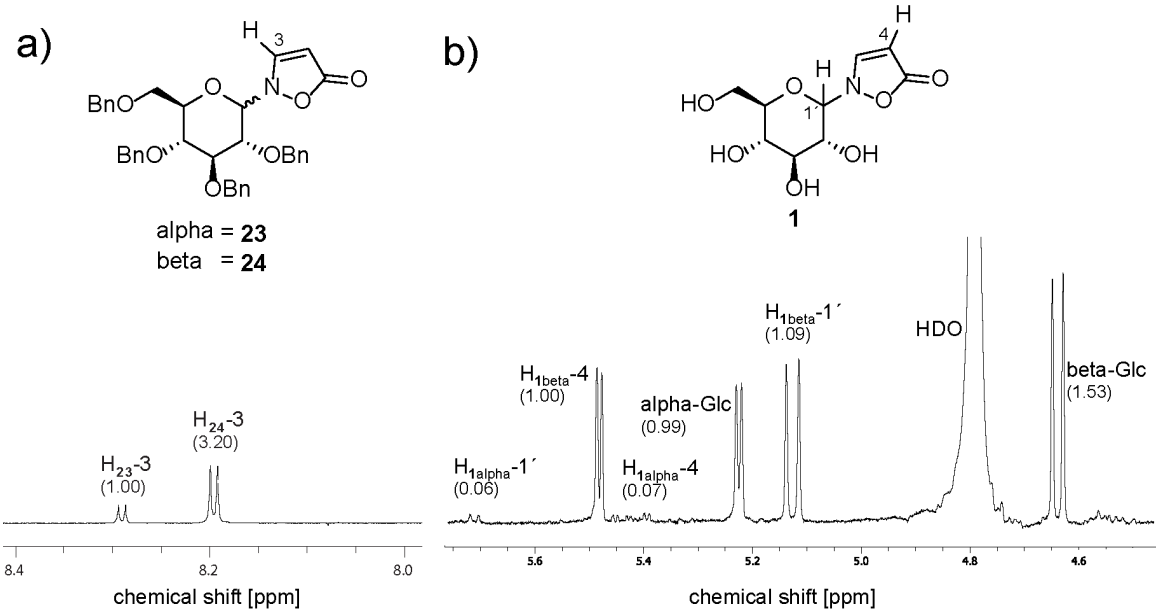


Figure 23 a) 500 MHz ^{1}H NMR spectrum of the reaction mixture providing compounds **23** and **24** in CD_3CN; b) 400 MHz ^{1}H NMR spectrum of a typical D_2O extract of the reaction mixture mainly providing compound **1**; The relative integrals are given in brackets.[77,80]

The NMR experiments show that the α/β-ratio in case of the protecting group strategy (Fig. 23 a) is 1:3.2 while in case of the alternative method the ratio equals 1:16 (Fig. 23 b), as proposed by the two different mechanisms (Fig. 22), depending on the structures of the starting materials (Fig. 23 a open-chain oxime, Fig. 23 b cyclic *N*-hydroxyglucopyranose). In addition, figure 23 b shows that glucose is formed in the aqueous reaction medium due to hydrolysis of the acylated *N*-hydroxyglucopyranose. This observation is an explanation for the limited the yields of the reaction in case of the protecting group free synthetic route. To circumvent this hydrolysis, the reactions were also performed in (less reactive) methanolic medium without success. When carried out in methanol as a solvent, the yields were smaller and the reaction times increased in case of glucose. Another observation from the NMR measurements, as indicated in figure 23 b, is that the consumption of the starting material (*N*-hydroxyglucopyransolamine) is nearly quantitative, if only 1.5 equivalents of DCC/PA were applied. This indicates that the hydrolysis occurs at the acylated *N*-hydroxyglucopyransolamine, rather than at the product of DCC and propynoic acid (see section 1.2.1. for a mechanism of DCC couplings).

Compared to the reactions carried out in acetonitrile, the reaction rate in the aqueous medium is much faster (several hours compared to 7 days). This indicates that a major rate limitation is due to the protonation at the C-4-position, rather than due to the attack of the nitrogen atom at the C-3-position of the triple bond.

While in case of the successfully transformed sugars glucose, maltose, xylose and fructose the rate of the sugar-ring-closure and the formation of the isoxazolin-5-one ring seem to overcome the rate of hydrolysis to a certain extent, it is hypothesized that the ribose derivatives do not show a first ring closure with a rate, which is high enough to overcome hydrolysis at all. This may refer to the *cis*-orientation of the vicinal hydroxyl-substituents. In case of 2-deoxyribose the missing 2-OH group (being present in all other applied sugars) may leads to higher hydrolysis rates at the C-1´-position, due to increased steric access.

The in this chapter presented results allow the simple and rapid synthesis of naturally occurring compounds **1** and **2** as well as other isoxazolin-5-one glycosides **26** to **29**. As a

consequence of this rapid access and due to the possibility to use unprotected glucose as a starting reagent for the construction of compounds **1** and **2**, it is reasonable to synthesize $^{13}C_6$-labeled compounds **1** and **2**, using commercial $^{13}C_6$-D-glucose. These stable-isotope-labeled compounds were synthesized applying the described techniques and used as internal standards for quantification experiments (see section 2.2.). Furthermore, the corresponding (physico-)chemical stability was investigated to get insights into possible biological roles and functions of the naturally occurring derivatives. The results of these experiments are discussed in the following section and reflected in section 2.2.2.

2.1.2. Photosensitivity and chemical properties

To investigate the effectivity of photodegradation as a general property of isoxazolin-5-one glycosides in water, solutions of the synthesized compounds **1** and **26** to **29** were prepared and irradiated with a weak UV handlamp at rt. Parallel to the irradiation of the sample, the UV absorption A around 260 nm was recorded. The same experiment was done using (commercial) uridine as a standard. By comparison of the degradation rates at the beginning of the irradiation, the quantum yields of this process were determined (Fig. 24).[80]

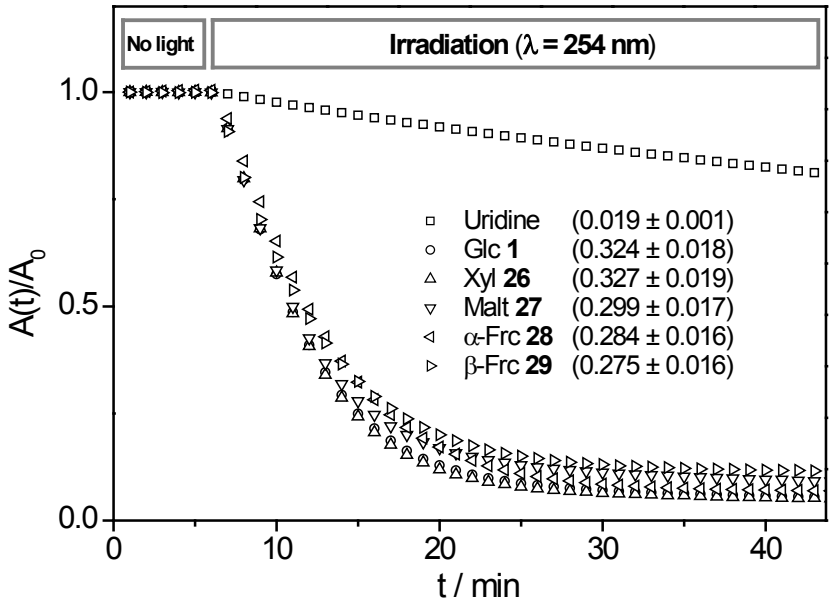


Figure 24 Photodegradation experiments using compounds **1** and **26** to **29** in H_2O; quantum yields ± estimated errors are given in brackets.[80,85]

In addition, ^{1}H NMR measurements of irradiated samples of the glycosides **1**, **28** and **29** showed quantitative formation of the corresponding free sugar, glucose and fructose respectively, after comparison with spectra of standard solutions. These results show that the isoxazolin-5-one moiety is a very effective leaving group in the anomeric position of glycosides upon irradiation at $\lambda \approx 260$ nm in water at rt and neutral pH. In contrast, these compounds do not show any observable change at very low pH values and rt, which is very unusual for any kind of glycoside.[4,80] Furthermore, compound **1** remained completely unaltered in a D_2O solution with β-glucosidase from almonds after days.[80] Thus, it is unlikely that the heterocycle can be cleaved at all under physiological conditions.

These results show that synthetic strategies, relying on either basic or acidic conditions do not yield significant amounts of isoxazolin-5-one glycosides. One reason is the instability of the heterocycle under acidic conditions, while on the other hand the *N*-substituted product is unstable at high pH. A second major drawback in terms of the synthesis of isoxazolin-5-one glycosides is the poor selectivity of the substitution reaction itself, allowing the heterocycle to be substituted not only at the nitrogen atom, but also at especially the oxygen and maybe other positions, as observed earlier.[2] Due to these findings, the isoxazolin-5-one derived compounds can be synthesized more efficiently as described by Baldwin and coworkers as well as in this work.[74-77,80] The following sections describe the application of authentic synthetic standards of compounds **1** and **2** to investigate the occurrence, amounts as well as the biosynthesis of these substances in juvenile Chrysomelina.

2.2. Biological role of compounds 1 and 2 in Chrysomelina Larvae

2.2.1. Protection of different life stages and chemotaxonomy

HPLC-MS analyses of samples from *Phaedon cochleariae* indicated the occurrence of isoxazolin-5-one derived glucosides **1** and **2** in the hemolymph of juvenile Chrysomelina. After comparison of these analyses with spectra of authentic synthetic standards of **1** and **2**, the natural compounds were confirmed to be present in the larval hemolymph, as shown via NMR and HPLC-MS (Fig. 25).

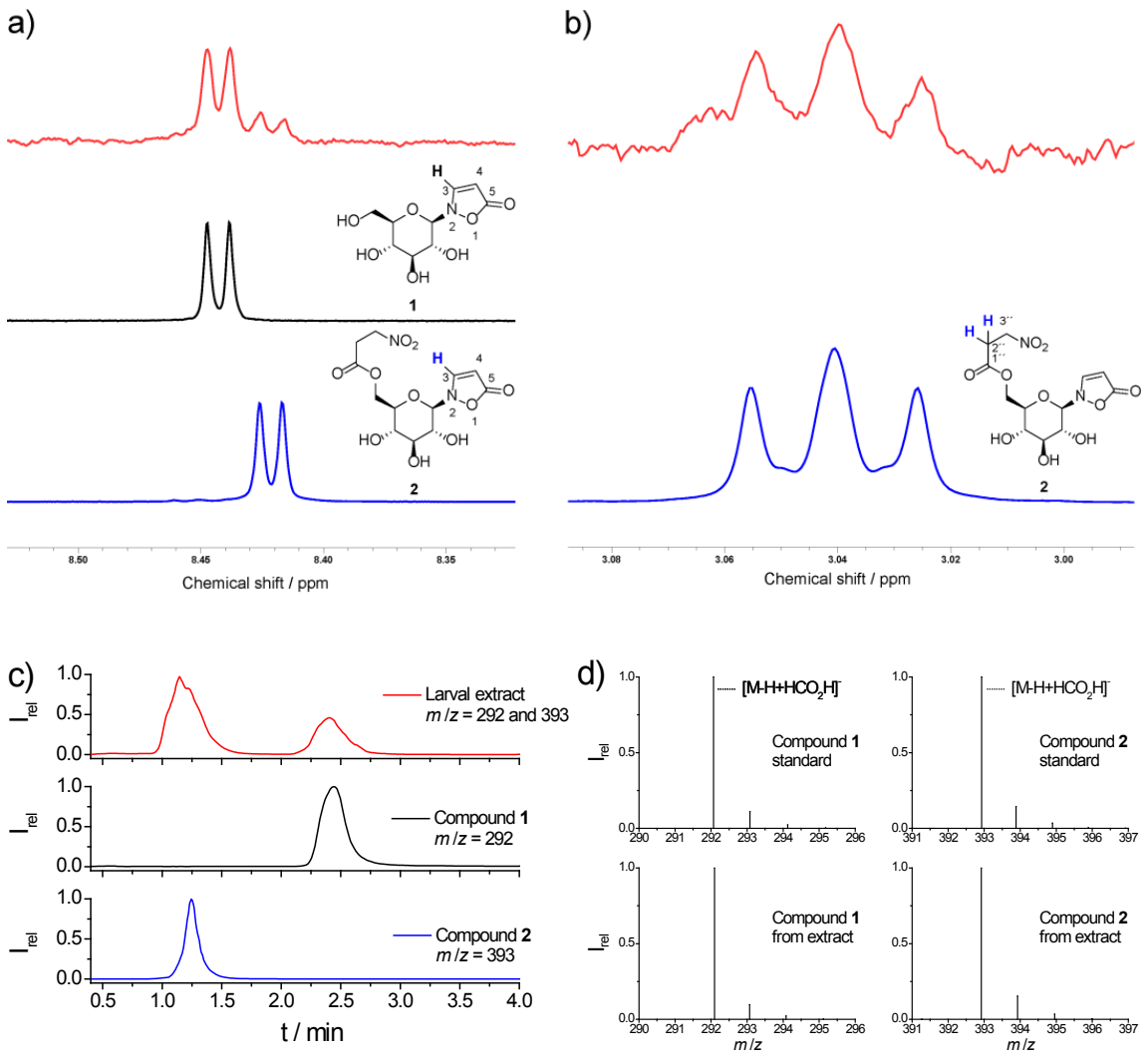


Figure 25 [1]H NMR spectra of hemolymph pooled from 30 *C. populi* larvae (red), compound **1** (black) as well as compound **2** (blue) in CD_3OD at 400 MHz; a) shows doublet signals of the H-3 position, b) shows the triplet of H-2´´; c) chromatograms of synthetic standards as well as larval extracts, measured on a LUNA-NH_2 column (Phenomenex), eluted with MeCN/H_2O (9:1, isocratic, 25°C) applying APCI; d) Mass spectra of synthetic standards of compounds **1** and **2** as well as from larval extracts; compounds **1** and **2** show addition of formic acid (HCO_2H) and loss of a proton ($[M-H+HCO_2H]^-$) under these conditions.

Further investigations were carried out with 17 different species of leaf beetles belonging to diverse subtribes of Chrysomelina, including Chrysomelini as well as Chrysolinini. In case of the Chrysomelini subtribe compounds **1** and **2**, as well as other 3-NPA esters have previously been identified in adult secretions and eggs, while in the latter group such derivatives were not detected.[15,86] In agreement with the first analyses on the hemolymph of *P. cochleariae* and *C. populi*, all species belonging to the Chrysomelini subtribe were

positively tested in terms of the occurrence of compounds **1** and **2** in the larval hemolymph. Due to the presence of compounds **1** and **2** in eggs, larvae and adult secretions of these insects, a chemotaxonomic allocation of leaf beetle species to their corresponding subtribes due to the presence of isoxazolin-5-one glucosides is obvious (Fig. 26).[87]

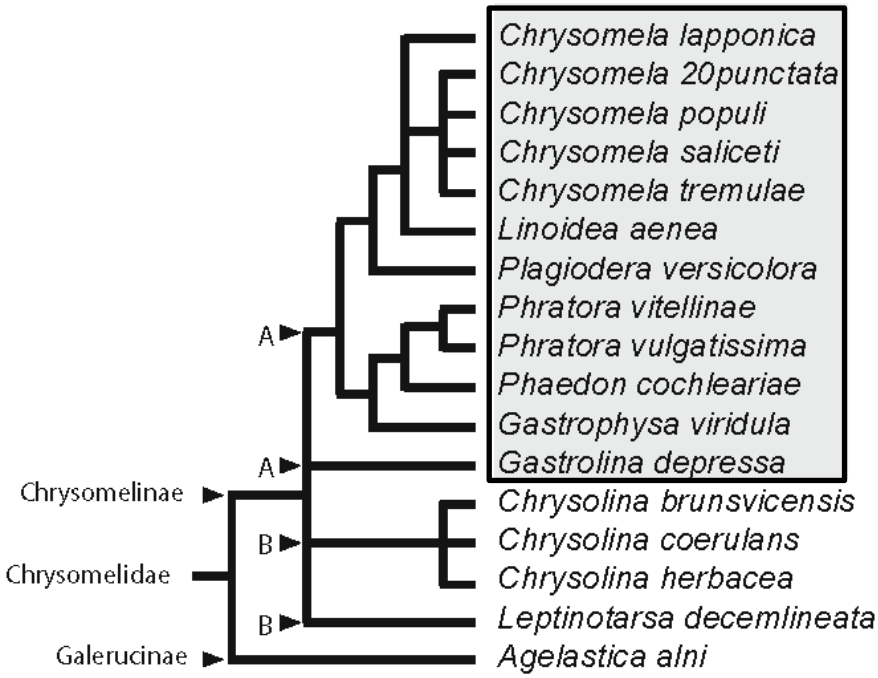


Figure 26 Phylogeny of leaf beetles (Chrysomelidae) correlated with the occurrence of isoxazolin-5-one glucosides **1** and **2**; species in the box contain compounds **1** and **2** and belong to the subtribe Chrysomelini, which is assigned with A; B allocates the subtribe Chrysolinini.[87]

Additional LC-MS analyses of whole pupa-extractions as well as adult hemolymph samples confirmed this conclusion (results of LC-MS analyses are analogous to the findings shown in Fig. 25). Furthermore and in contrast to adult chrysomeline leaf beetles, isoxazolin-5-one glucosides were not detected in the larval defensive secretion and thus are not transported parallel to other glucosides, e.g. salicin in *C. populi*, into the secretory system, although both types of glycosides occur in the hemolymph of juvenile Chrysomelina.[87] In addition, multi-ester compounds or 3-NPA esters lacking the isoxazolin-5-one heterocycle that are major components of adult secretions were not detected in case of the larvae at all, neither in NMR- nor in HPLC-MS-analyses. These

results indicate that isoxazolin-5-one and 3-NPA moieties exclusively occur in compounds **1** and **2** in juvenile Chrysomelina. Thus, it should be sufficient to address compounds **1** and **2** for quantitative investigations on isoxazolin-5-one and 3-NPA moieties in leaf beetle larvae.

To determine the amount and estimate the ecological importance of these compounds for Chrysomelina, first quantifications of isoxazolin-5-one glucosides **1** and **2** in larval hemolymph samples were carried out by HPLC-MS analyses based on external synthetic standards.[87] These experiments revealed concentrations of compounds **1** and **2** of up to 50 nmol/mg hemolymph fresh weight, which is equivalent to concentrations in the range of 50 mmol/l, if it is considered that 1 mg hemolymph equals a volume of 1 µl. Due to the rapid synthetic access to $^{13}C_6$-labeled compounds **1** and **2** via commercial $^{13}C_6$-glucose (compare section 2.3.1.), it was possible to spike the samples with defined amounts of authentic internal standards for quantification experiments.[77,80,88] This SIL-IS-method minimizes the matrix effects that are significant in HPLC-MS analyses of natural samples.[89] At the same time an external calibration is redundant. This reduces the time consumption for the analyses and increases the reproducibility significantly.[89]

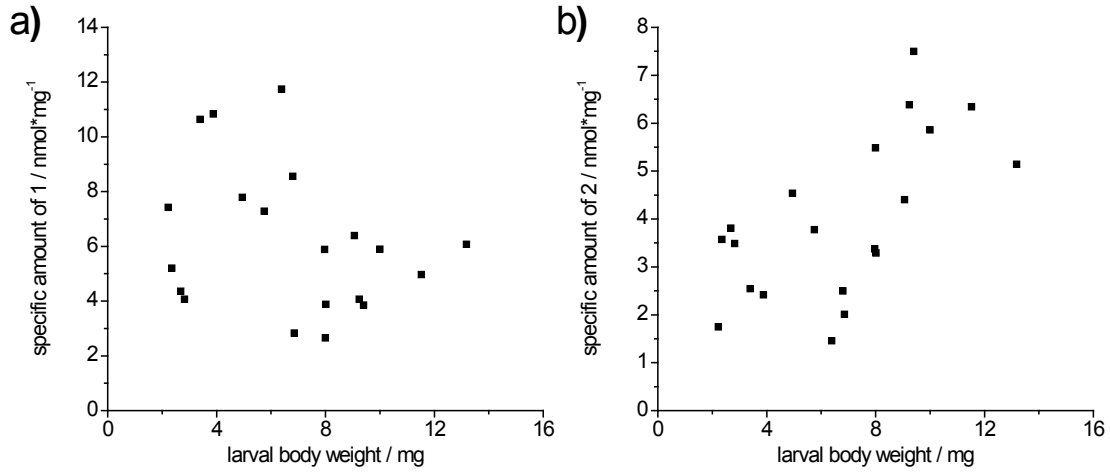


Figure 27 Specific amounts[1] of compounds **1** (a) and **2** (b) in *P. cochleariae* larvae with respect to the body weight in nmol/mg, determined by HPLC-MS using SIL-IS; N_{biol}=20.[88]

[1] The molar amount per mass is defined here as the specific amount.

Applying this SIL-IS approach, the amounts of compounds **1** and **2** in juvenile Chrysomelina were determined per larval body weight (Fig. 27).[88] The above shown results are consistent with previously published data, where total concentrations of compounds **1** and **2** above 10 mmol/l were described in case of eggs of Chrysomelina.[15] This indicates that leaf beetle larvae are chemically protected by the 3-NPA derived ester compound **2** as well. This protective function of compound **2** as a pre-toxic compound is understood via hydrolysis of the ester bond upon digestion of predated leaf beetle larvae, resulting in the release of the actual toxin 3-nitropropanoic acid (3-NPA). This leads to an intoxication of the predator with 3-NPA, as indicated by former experiments using ants (*Myrmica rubra*) as potential predators. In these experiments, an increased mortality, comparable with starving ants, was observed.[15] In addition, a significant repellent effect of compound **2** in binary choice tests was demonstrated.[15] These results indicate the ecological significance of the 3-NPA ester **2** as a pre-toxic and repellent secondary metabolite in leaf beetle larvae as well. Thus, two defensive mechanisms in the Chrysomelini subtribe are active at the same time, as represented by repellent excreted volatiles in the secretions and the 3-NPA-ester **2** in the hemolymph.[24,25,27,87]

Furthemore, an increase of the content of compound **2** and thus of 3-NPA production with respect to the larval body weight was observed.[87] Applying the SIL-IS-approach, this increase is statistically significant for linear regression (R^2=0.400, P=0.003, N_{biol}=20, N_{tech}=1), although the number of measurements per biological replicate was reduced by the factor of 4, in comparison to quantification by external calibration (R^2=0.345, P<0.001, N_{biol}=30, N_{tech}=1).[87] This increase of the specific amount of 3-NPA-units per larval body weight indicates autogenous production of this moiety, which is discussed in more detail in section 2.3.

2.2.2. Functions of non 3-NPA-containing compound 1

While the role of compound **2** is principally understood as a non-toxic storage molecule for the actual toxin 3-NPA, the biological function of compound **1** remained unknown in Chrysomelina as well as in plants producing this compound. Injections of [1-^{13}C,^{15}N]-3-nitropropanoic acid into the larval hemolymph show the subsequent conjugation of compound **1** with the toxin, resulting in the formation of compound **2** (Fig. 28).[87]

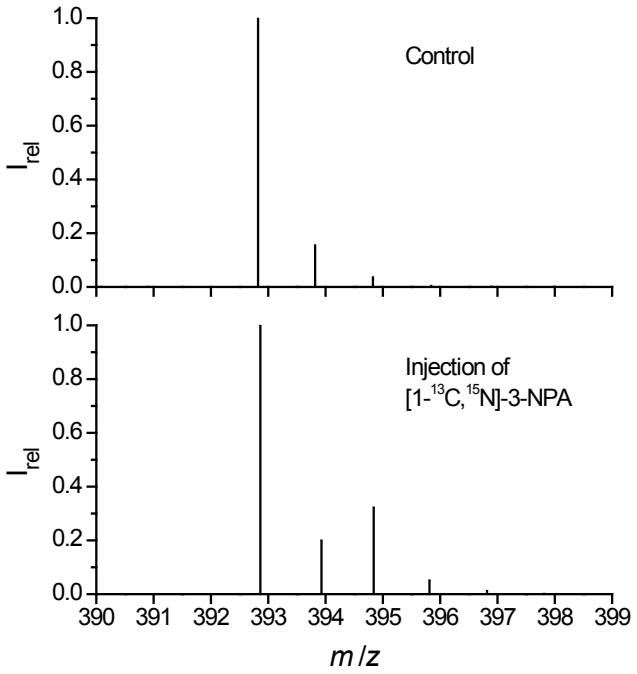


Figure 28 Mass spectra of *C. populi* hemolymph samples after injection of buffer (top) and [1-^{13}C,^{15}N]-3-NPA (bottom).[87]

Furthermore, the specific amount of compound **1** often exceeds the amount of compound **2**, especially in the early stages of the larvae (Fig. 27, previous section).[88] With increasing larval body weight, the specific amount of compound **2** significantly increases, while the amount of compound **1** slightly decreases (compare section 2.2.1.). These results indicate that compound **1** serves as a platform for the prevention of self-intoxication with free 3-NPA. Although this hypothesis is plausible, it was of interest, whether compound **1** itself shows some cytotoxic effects. Since antifungal properties have been previously shown in case of components of larval secretions[90,91] and some non-natural isoxazolin-5-one derivatives **11-13** (section 1.1.2.),[60] the cytotoxicity of compound **1** was tested using cell cultures of microorganisms as well as human cell lines. For this purpose, solutions of isoxazolin-5-one glucoside **1** were added to cell lines of phyto- and entomopathogens as *Beauveria Bassiana*, *Conidiobolus coronatus*, *Alternaria alternata*, *Fusarium graminearum*, *Cladosporium herbarium* and *Glomerella cingulata* as well as the human pathogens *Bacillus subtilis*, *Staphilococcus aureus*, *Escherichia*

coli, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Mycobacterium vaccae*, *Sporobolomyces salmicolor*, *Candida albicans* and *Penicillium notatum*. These experiments were performed by Christiane Weigel (Group of Kerstin Voigt at the HKI in Jena). Furthermore, human HUVEC, K-562 as well as HeLa cell lines were tested. These experiments were performed by Dr. Hans-Martin Dahse.

All of those tests resulted in no detectable inhibition of growth or increased mortality in the cell lines treated with solutions of compound **1**, applying concentrations of 0.1 to 1 mg/ml (4 to 40 mmol/l). These results are consistent with the lack of toxicity of compound **1** against ants (*Myrmica rubra*), shown by Pasteels and coworkers.[15] Due to the high chemical stability of the isoxazolin-5-one ring bound to the glycosidic position in compound **1** (section 2.1.2.), a cleavage of the C-N bond to release the heterocycle **17** into the solution, analogous to the cleavage of the 3-NPA ester, is unlikely. In addition, the free heterocycle **17** is unstable in aqueous solution, forming cyano acetic acid as a degradation product, which is not known to elicit any toxic effects.[73]

To summarize these results, isoxazolin-5-one glucoside **1** circulates in millimolar concentrations in the hemolymph and is non-toxic to the insect itself as well as to a variety of other cell types from different organisms. The capacity for esterification of **1** with the CoA ester of 3-NPA is always sufficient to prevent the leaf beetle larvae from poisoning with this irreversible mitochondrial inhibitor. Thus, it can be assumed that a major benefit of compound **1** for leaf beetles is the prevention from self-intoxication with 3-NPA via formation of the corresponding ester **2**.

2.3. Biosynthesis of compounds 1 and 2 in Chrysomelina larvae

2.3.1. Synthesis of standards and putative metabolic intermediates

In order to study the metabolic pathway of the biosynthesis yielding compounds **1** and **2** in Chrysomelina, several stable-isotope-labeled putative intermediates were synthesized for trace experiments.[92] As proposed earlier[6,32,93,94] as promising intermediates in such pathways, it was of interest to access labeled *N*-oxidized derivatives of β-alanine, e.g. compounds **30** and **31** as well as [1-^{13}C,^{15}N]-3-NPA **8**. To realize the syntheses of compounds [1-^{13}C,^{15}N]-**8** as well as [1-^{13}C,^{15}N]-**30** a synthetic route described by Baxter and coworkers[37] was applied, introducing an additional ^{15}N-label (Fig. 29 a).

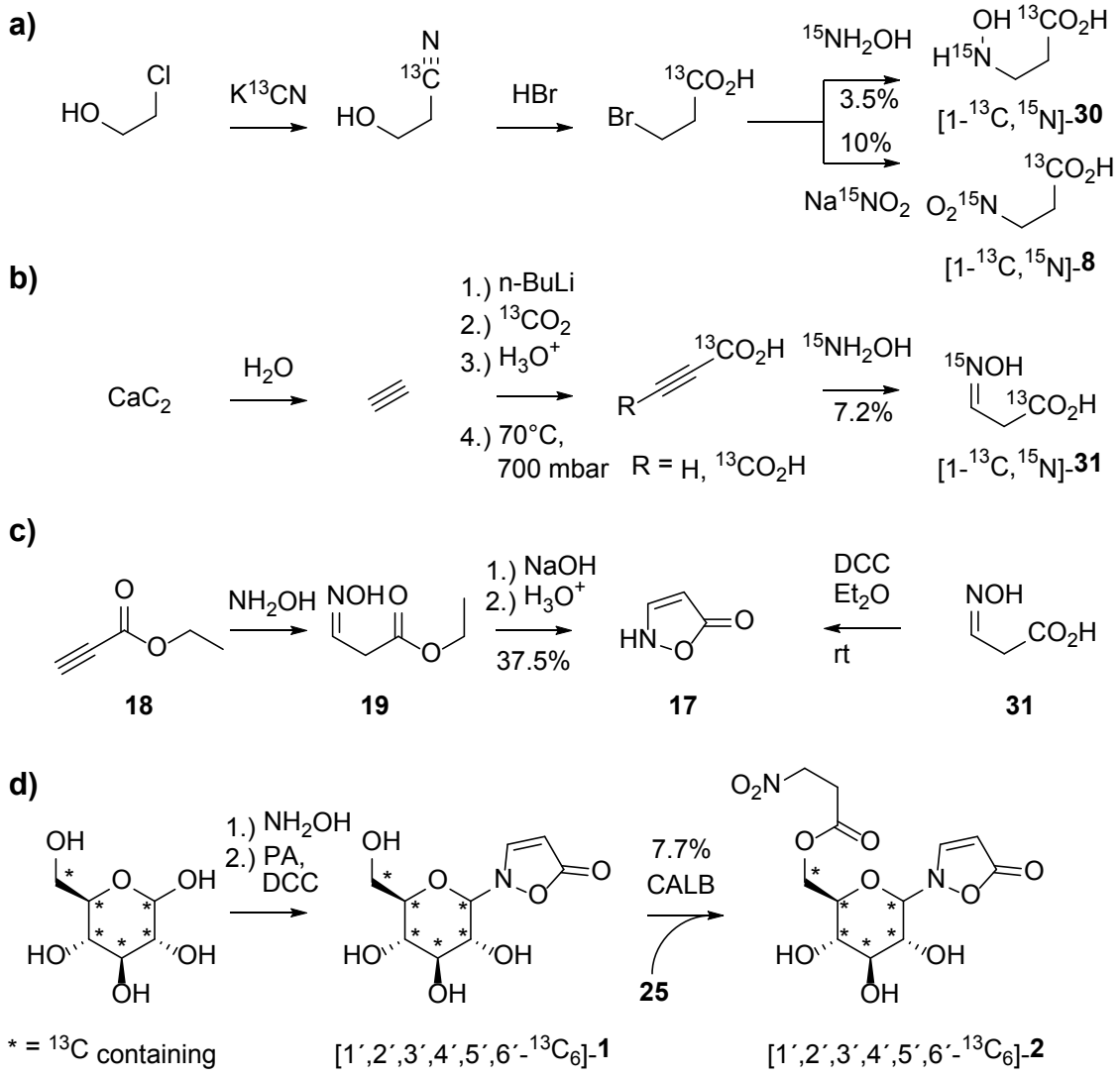


Figure 29 Conditions for the syntheses of intermediates, used to study the metabolic pathway of compounds **1** and **2** in Chrysomelina larvae; total isolated yields are given; for details see lit.[37,73,76,77,80,88]

Two labeled atoms at defined positions provide major advantages compared to a single label or to a deuteration of acidic CH-positions. This is due to the lower abundance of naturally occurring double-labeled ([M+2]) compared to single-labeled ([M+1]) products. In case of deuteration, the partial re-exchange with ^{1}H under physiological conditions yields a randomly distributed pattern of differently deuterated products, reducing the sensitivity of measurements as well as the ease of interpretation of the obtained spectra.

For the synthesis of compound [1-^{13}C,^{15}N]-**31**, [1-^{13}C]-propynoic acid was synthesized as an intermediate according to a modified procedure described by Baldwin and coworkers.[76] [1-^{13}C]-propynoic acid was then further transformed into [1-^{13}C,^{15}N]-**31** by addition of $^{15}NH_2OH$ (Fig. 29 b). To increase the yield for the synthesis of [1-^{13}C]-propynoic acid, the reaction mixture was intermediately heated under reduced pressure, to force the decarboxylation of the side product acetylene dicarboxylate, yielding a higher content of the desired monoacid [1-^{13}C]-propynoate. In general, the overall yields of these transformations are rather small, which limits the application of the compounds for biosynthetic investigations. However, the isolated amounts of the products were sufficient for multiple injection experiments (see section 2.3.2.). Column chromatography provided appropriate purity (for details see original publication[88]).

To test whether the heterocycle **17** itself occurs as an intermediate in the biosynthesis of compounds **1** and **2** in Chrysomelina, a synthesis of [1-^{13}C,^{15}N]-labeled isoxazolin-5-one **17** via oxime **31** and DCC in ether was performed (Fig. 29 c). After this attempt, the column chromatography (SiO_2 and RP-18) failed to isolate heterocycle **17**, although UV activity at appropriate wavelengths (around 260 nm) of these solutions could be measured prior to column chromatography. This observation might correlate with the instability of compound **17** under acidic conditions. To circumvent this problem of isolating small amounts of an (expensive) labeled product, the unlabeled heterocycle **17** was synthesized and isolated by extraction, according to De Sarlo *et al.*[73] The isolated product **17** was then used for *in vitro* investigations using larval tissue samples (see section 2.3.2.).

For quantitative studies, the synthesis of stable-isotope-labeled compounds **1** and **2** was carried out (Fig. 29 d).[77,80,88] With the use of these six-fold-labeled substances, quantification of natural compounds **1** and **2** from biological samples was possible using the SIL-IS[89] approach, as described in sections 2.2 and 2.3.2.

2.3.2. Metabolic pathway

The previously described synthesized compounds as well as commercially available ^{13}C- and ^{15}N-labeled substances were applied to larvae of *P. cochleariae*, *C. populi* and *G. viridula* to investigate the metabolic pathway for the formation of compounds **1** and **2** in juvenile Chrysomelina. In first experiments, commercial [$^{13}C_4$,^{15}N]-aspartate,

[$^{13}C_3$,^{15}N]-β-alanine, [$^{13}C_3$]-propanoate as well as [$^{13}C_5$,^{15}N]-valine were fed to larvae of *P. cochleariae* and LC-MS analyses were performed from MeCN/H_2O-extracts of the so treated juvenile leaf beetles (Fig. 30).[88]

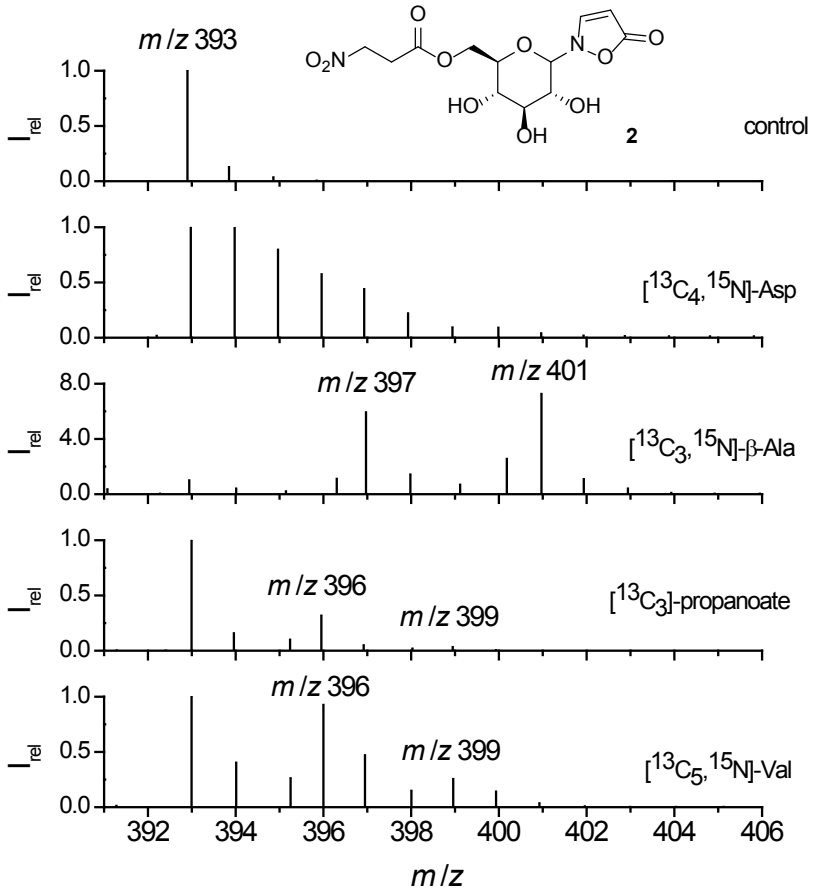


Figure 30 Representative mass spectra after LC-separation of larval extracts from *P. cochleariae* feeding on the given substances for 10 d.[88]

The mass spectra indicate that randomly re-assembled fragments of [$^{13}C_4$,^{15}N]-Asp are incorporated into compound **2** as evidenced by an increase of the intensity of isotopic peaks at *m*/*z* 394, 395, 396, 397, 398, 399 and 400 compared to the control spectrum. These signals at *m*/*z* 394 to 400 exhibit an approximately linear decrease in their intensity with ascending *m*/*z* ratio. In contrast, the spectrum of larval samples treated with [$^{13}C_5$,^{15}N]-Val show signals of distinct ions of maximum intensity at *m*/*z* 396 as well as 399, indicating an incorporation of intact fragments deriving from this amino acid into compound **2**. These observations provide clear evidence for a direct pathway from the

essential amino acid valine to compound **2**, while the incorporation of aspartate seems to derive from general multistep metabolic pathways within these organisms. Observations analogous to the results upon feeding of [$^{13}C_5$,^{15}N]-Val were made in case of the [$^{13}C_3$,^{15}N]-β-alanine and [$^{13}C_3$]-propanoate treated larvae, as indicated by distinct peaks at *m*/*z* 397 and 401 as well as 396 and 399 respectively. In addition, the distinct signals at *m*/*z* 399 (valine and propanoate) as well as 401 (β-alanine) can be explained by incorporation of two consecutive units into compound **2**, deriving from these precursors. This indicates that both, the isoxazolin-5-one as well as the 3-NPA moiety, derive from the same precursors, as exemplified using valine, propanoate as well as β-alanine. This hypothesis is further evidenced by the mass spectra of compound **1** after LC-separation of the larval samples as well as LC-MS analyses of hydrolyzed compound **2**, which resulted in the detection of labeled free 3-NPA (for details see literature[88], ESI).

The spectra measured from larval samples treated with [$^{13}C_5$,^{15}N]-Val additionally show increased intensities at *m*/*z* 394, 395, 397, 398 and 400, compared to the control spectrum. This observation can be explained by essential deamination and consecutive transamination, transferring the ^{15}N-atom to natural unlabeled intermediates, leading to peaks at *m*/*z* 394 and 395 or to labeled intermediates, leading to an increase of signals at *m*/*z* 397, 398 and 400.

As a consequence of these findings, intermediates derived from β-alanine, such as compounds **8**, [1-^{13}C,^{15}N]-**8**, **17**, [1-^{13}C,^{15}N]-**30** and [1-^{13}C,^{15}N]-**31** were applied to larval fat body tissue (in case of **8** and **17**) or were injected into the larval hemolymph (in case of [1-^{13}C,^{15}N]-**8**, [1-^{13}C,^{15}N]-**30** and [1-^{13}C,^{15}N]-**31**) to explore further steps in the metabolic pathway. All of these experiments resulted in positive incorporation of the putative intermediates, as shown in Fig. 31.[88]

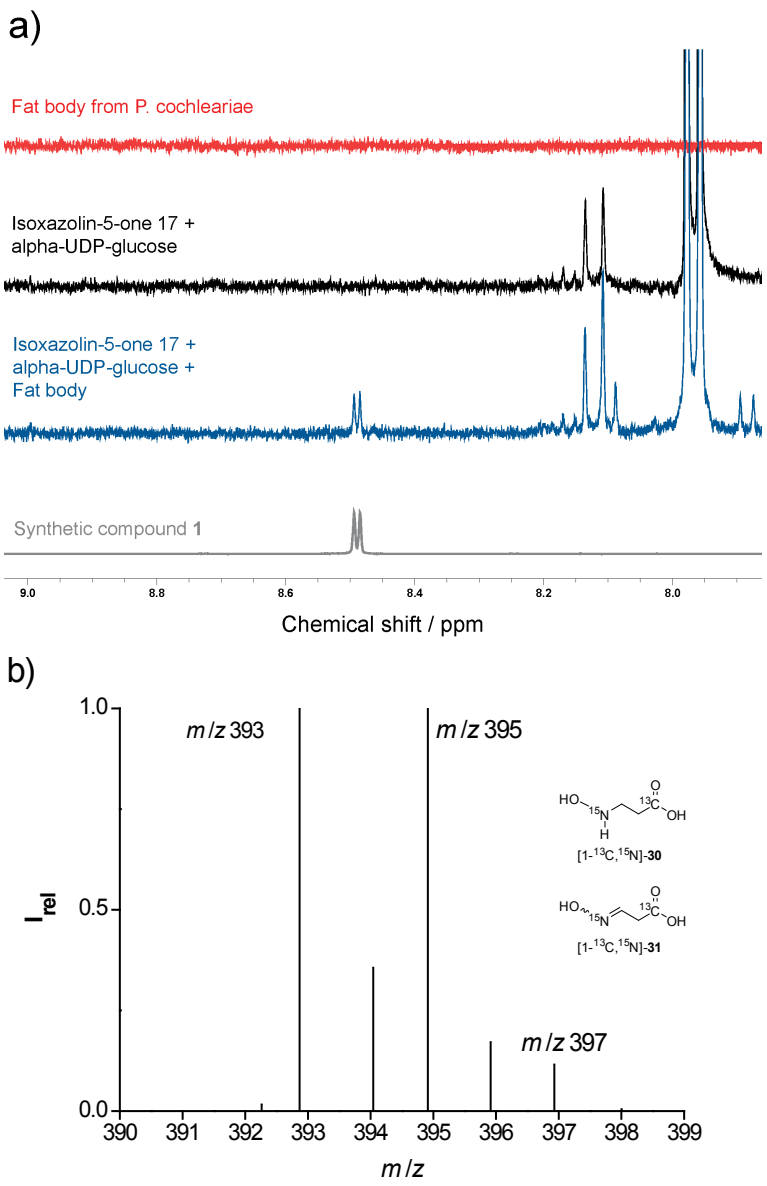


Figure 31 a) Representative ^{1}H NMR spectra of the supernatant of a fat body suspension from *P. cochleariae* (red), compound **17** and α-UDP-glucose (black); supernatant of fat body from *P. cochleariae*, compound **17** and α-UDP-glucose (blue) and synthetic compound **1** (grey); b) Representative mass spectrum of compound **2** after LC separation of a larval extract from *P. cochleariae*, injected with either compound [1-^{13}C,^{15}N]-**30** or -**31**; for a control spectrum see Fig. 30.[88]

The above shown results lead to a novel supposed biosynthetic pathway for the formation of compounds **1** and **2** in Chrysomelina larvae, starting from essential amino acids as demonstrated for valine (Fig. 32).

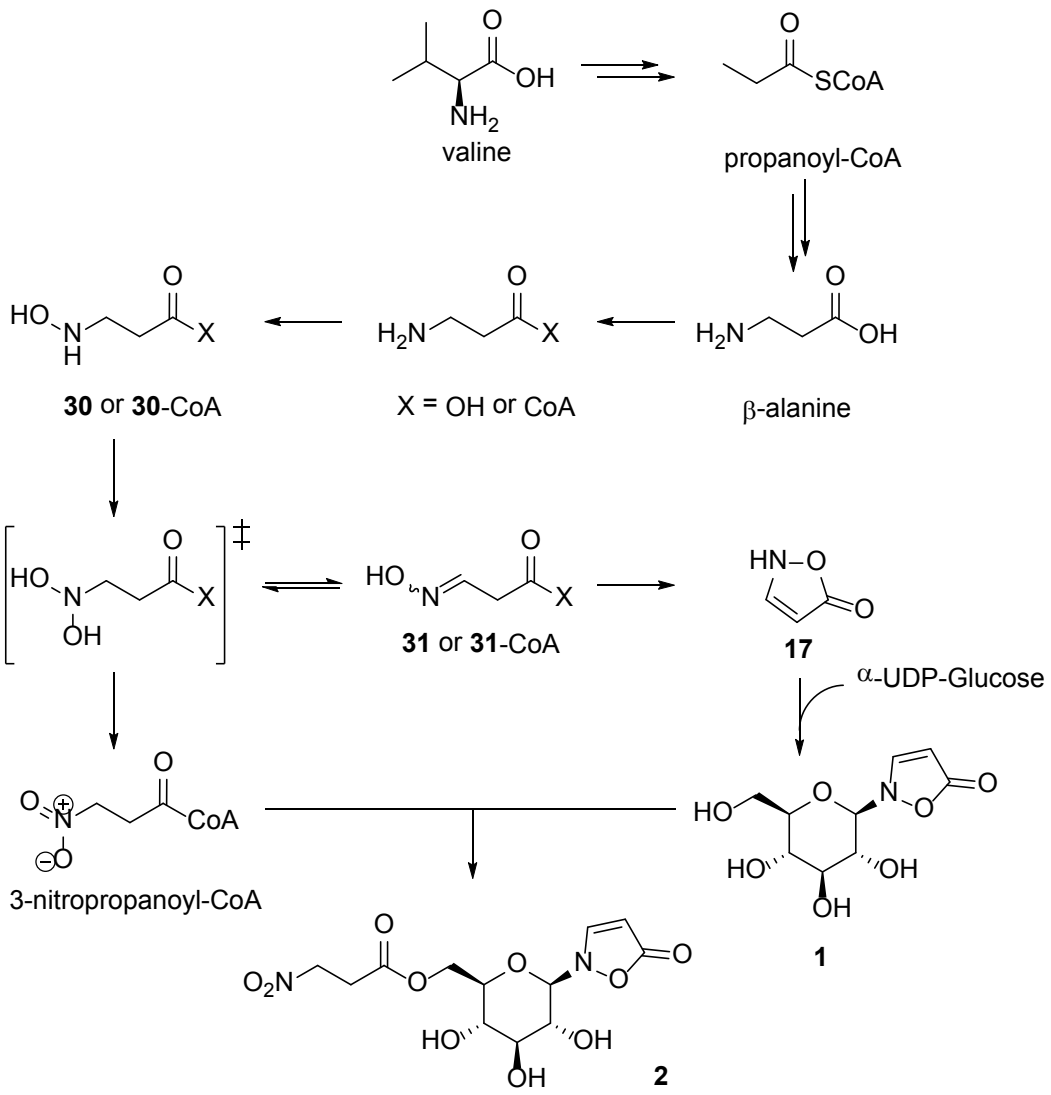


Figure 32 Proposed metabolic pathway for the biosynthesis of compounds **1** and **2** in juvenile Chrysomelina.[88]

It is supposed that over several steps valine is converted into propanoyl-CoA, being described as a general occurring catabolic pathway for several essential amino acids.[95-97] Propanoate can then be transformed into β-alanine upon oxidation, followed by transamination or addition of ammonia. β-Alanine is then oxidized at the nitrogen atom in three consecutive steps, most likely upon monooxygenation, to form 3-NPA. The formation of the heterocycle proceeds via two oxidation steps, resulting in the formation of the oxime **31**, which cyclizes either enzymatically or non-enzymatically to form isoxazolin-5-one **17**. In the presence of α-UDP-glucose, the heterocycle **17** is then finally

transformed into glucoside **1**. Under consumption of ATP and Coenzyme A, compounds **1** and 3-NPA are transformed into the nitro-ester **2**. To study the contribution of the described intermediates to the biosynthesis of compounds **1** and **2** in juvenile Chrysomelina, the percentiles of incorporation of the injected compounds in case of [$^{13}C_4$,^{15}N]-aspartate, [$^{13}C_3$,^{15}N]-β-alanine as well as [$^{13}C_3$]-propanoate were determined using *P. cochleariae* larvae (Fig. 33, details are described in the original publication[88]).

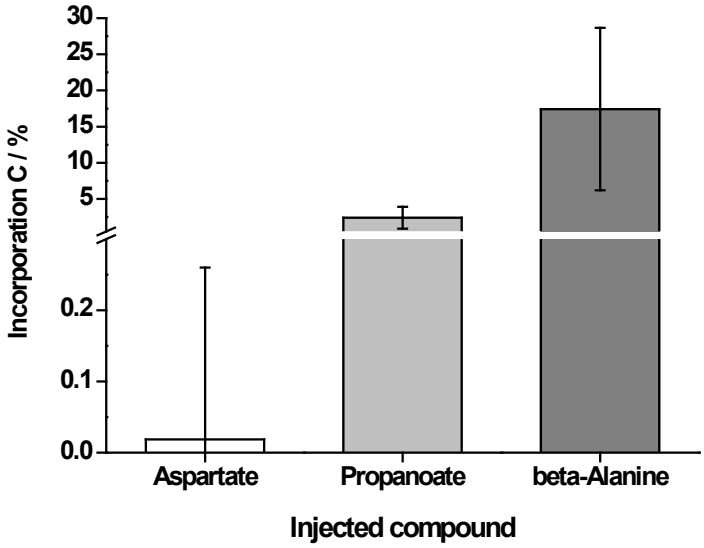


Figure 33 Incorporations (C in %) of the injected substances into compounds **1** and **2** in *P. cochleariae*.

The results clearly indicate the low contribution of aspartate, while propanoate and especially β-alanine show much higher incorporation of up to 40%.[88] The incorporation of valine was estimated by comparison of the results of the feeding experiments after feeding on leaves coated with aspartate- and valine-solutions (analogous to literature methods[88], equ. 3). These analyses show that the incorporation of valine is 8.1 times higher than that of aspartate, indicating that Val is a significantly more important source than Asp for the construction of compounds **1** and **2** in juvenile Chrysomelina. Previously, it was demonstrated that free valine occurs in higher amounts in *Brassica rapa pekinensis* leaves compared to aspartate, which supports this hypothesis in case of *P. cochleariae*.[98] In addition, all three investigated species (*P. chochleariae*, *C. populi*

and *G. viridula*) show a very similar total content of isoxazolin-5-one and 3-NPA moieties with respect to their body weight (Fig. 34).

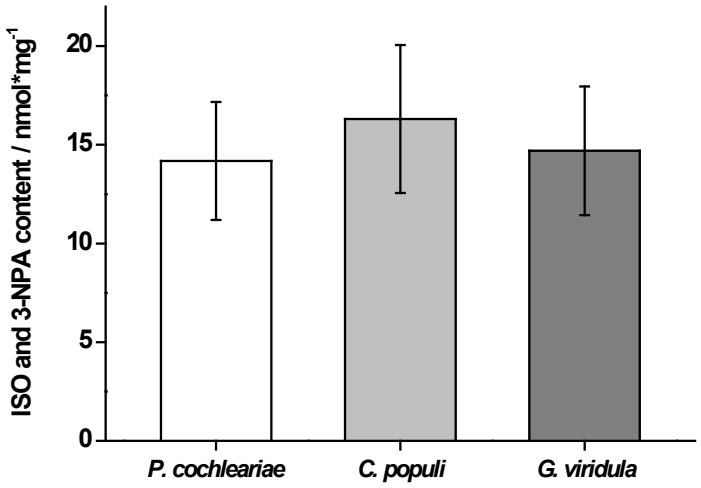


Figure 34 Isoxazolin-5-one and 3-NPA content in Chrysomelina species in nmol/mg, determined with SIL-IS; $N_{P.\ cochleariae} = N_{G.\ viridula} = 20$, $N_{C.\ populi} = 16$.

This further indicates a biosynthesis via generally occurring precursors, as essential amino acids, e.g. valine, as well as other host plant-derived nutrients, as β-alanine and propanoic acid. In (*dqf*)-COSY-NMR measurements of crude hemolymph samples from juvenile Chrysomelina a high content of free valine is indicated (Fig. 35).[87]

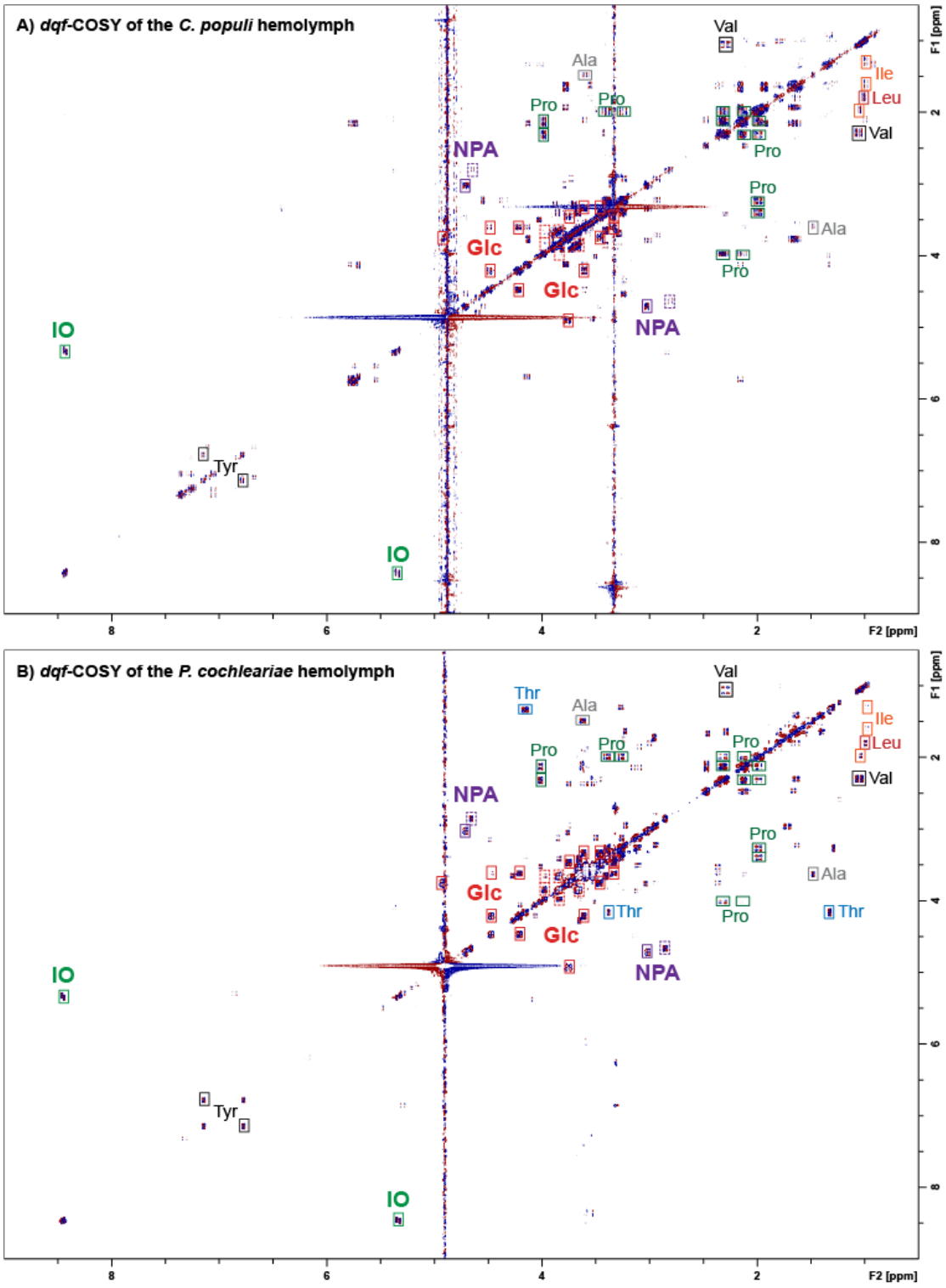


Figure 35 Double quantum filtered (*dqf*)-COSY spectra at 500 MHz in CD_3OD of the crude hemolymph from *C. populi* (**A**) and *P. cochleariae* (**B**); Signals corresponding to amino acids (three letter codes), as well as isoxazolin-5-one (IO), glucose (Glc), and 3-nitropropionate (NPA) units are labelled.[87]

These spectra provide evidence for the required efficient uptake of significant amounts of valine and other amino acids upon ingestion and digestion of the host plant leafs, needed for the biosynthesis of compounds **1** and **2** in Chrysomelina larvae.

No increase in isotopic peak intensities in compounds **1** and **2** occurred after feeding of leaves covered with [$^{13}C_2$]-malonate solutions. The same result was observed in case of threonine. In case of α-alanine only an incorporation of the nitrogen atom was observed upon feeding of leaves covered with [$^{13}C_3$,^{15}N]-α-alanine- and [^{15}N]-α-alanine-solutions. These results rule out alternative carbon sources for β-alanine, as described elsewhere[61,99] and indicate that the amino-nitrogen of proteinogenic amino acids serves as the nitrogen source.

The novel findings reveal that the formation of both moieties, the isoxazolin-5-one ring and 3-NPA, derives from the same precursors. A previously suggested pathway via decarboxylation of aspartate, providing required β-alanine, is unlikely due the incorporation of randomly reassembled fragments of aspartate, rather than intact atomic groups. Thus, essential amino acids as valine are ultimate precursors for the formation of compounds **1** and **2** rather than metabolites deriving from aspartate metabolism.

3. Summary

3.1. Synthesis of isoxazolin-5-one glucosides

An alternative access to isoxazolin-5-one glycosides was realized, based on a cascade reaction.[74,75,77,80] The total yield for the synthesis of naturally occurring compound **1** was improved from 1%[4] (Koenigs-Knorr) to 22%.[4,77,80] In a subsequent step, compound **2** could be synthesized by transesterification (Fig. 36).[77]

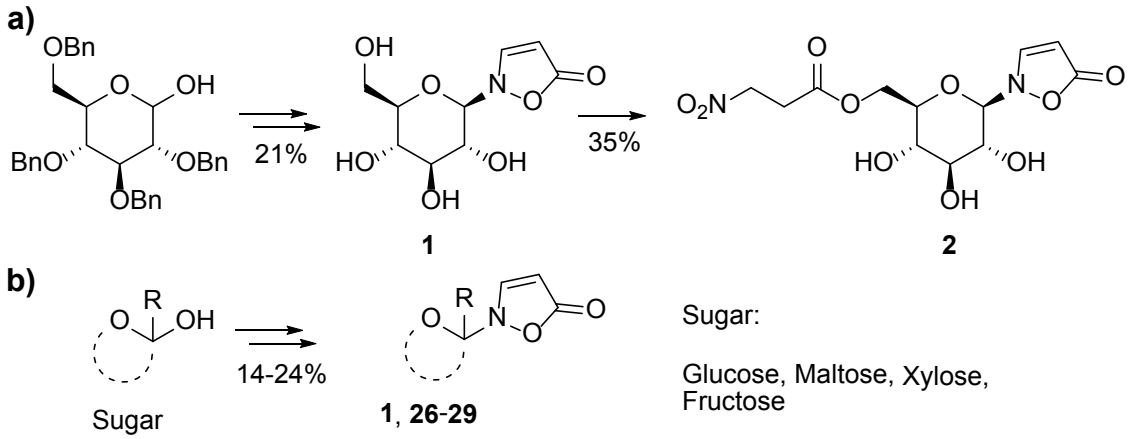


Figure 36 a) Novel approach for the synthesis of compounds **1** and **2**, starting from tetra-*O*-benzyl glucose; b) Principal for the synthesis of isoxazolin-5-one glycosides **1** and **26-29** from unprotected sugars as precursors.[77,88]

The required number of steps for the synthesis was further reduced by starting from unprotected sugars that were transformed into the corresponding oximes.[80] Using this method, compound **1** as well as four novel isoxazolin-5-one glycosides, derived from xylose, maltose and fructose were synthesized, purified and characterized.[80] Crystals of compound **1** could be isolated to perform a single-crystal x-ray diffraction analysis. All isoxazolin-5-one glycosides showed high quantum yields around 0.3 for photodegradation upon irradiation at a wavelength of 254 nm in aqueous media and rt, analogous to similar natural occurring isoxazolin-5-one derivatives.[4,80] These photoreactions resulted in quantitative release of the corresponding sugars.[80] Compound **1** shows high stability in presence of β-glucosidase from almonds.[80]

3.2. Ecological relevance of isoxazolin-5-one derivatives in leaf beetles

NMR- and HPLC-MS-based analyses of samples from juvenile Chrysomelina leaf beetles provided evidence for the occurrence of isoxazolin-5-one and 3-NPA derived glucosides that were only known from adult secretions as well as eggs of these species.[5,15] Upon comparison of spectra from authentic synthetic standards of these isoxazolin-5-one glucosides with the spectra of biological samples, the occurrence of compounds **1** and **2** was confirmed in Chrysomelina larvae, pupae as well as adult hemolymph.[87] Quantitative analyses revealed ecologically significant specific amounts of compounds **1** and **2** in the range of several nmol/mg, which correlates with millimolar concentrations.[88] The use of the SIL-IS approach significantly reduces the required time for analyses, while the analytical precision and accuracy are optimized.[89] The increase of the specific amount of compound **2** with respect to the body weight in Chrysomelina larvae was observed.[87,88]

The major biological function of compound **1** in Chrysomelina leaf beetles was identified to be the precursor for esterification with the toxin 3-nitropropanoic acid to form compound **2**, as a storage molecule that can circulate in the insect's hemolymph.[87] This hypothesis is supported by the high concentrations of compound **1** in the hemolymph, guaranteeing sufficient transesterification potential. Furthermore, no toxicity of this substance was observed upon application of significant concentrated solutions to diverse cell lines of fungi, bacteria as well as human cells.

The observed detoxification pathway in 3-NPA producers via ester-formation has not yet been described so far. Furthermore, these findings extend the understanding of chemical defense in juvenile Chrysomelina leaf beetles, showing toxin production as well as aposematic warning via secreted volatiles as two different modes of defense occurring at the same time.[24,25,87]

3.3. Biosynthesis of isoxazolin-5-one and 3-NPA moieties in Chrysomelina

Isotopic labeled and non-labeled putative intermediates of the biosynthetic pathway of compounds **1** and **2** in Chrysomelina larvae were applied to the insects or to suspensions of their fat body. HPLC-MS- as well as NMR-analyses of these samples resulted in the suggestion of an unexpected metabolic route for the formation of isoxazolin-5-one glucosides **1** and **2** in juvenile Chrysomelina (Fig. 37).[37,61,88]

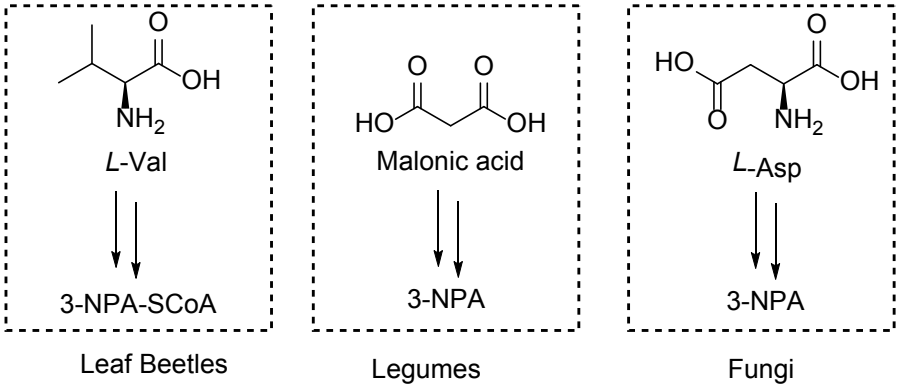


Figure 37 Comparison of different proposed biosynthetic pathways of 3-NPA.

The novel supposed pathway starts from essential amino acids, e.g. valine, and proceeds via propanoyl-CoA and β-alanine as intermediates. Further stepwise monooxygenation at the nitrogen atom of β-alanine leads to the formation of intermediate 3-(hydroxyamino)propanoic acid **30** as well as 3-(hydroxyimino)propanoic acid **31**. The oxime **31** is cyclized to form isoazolin-5-one **17**, which is transformed into compound **1** via α-UDP-glucose. Further oxidation of the oxime provides 3-nitropropanoic acid. The corresponding CoA-ester is finally transformed into compound **2**. Quantitative experiments revealed the significance of the proposed intermediates of the biosynthetic pathway. These experiments show that the isoxazolin-5-one as well as the 3-NPA moieties derive from the same precursors in Chrysomelina larvae. Malonate and threonine do not show incorporation.[88] A direct decarboxylation of aspartate to yield β-alanine was not observed.[6] Thus, the novel supposed pathway is alternative to the previously described metabolic routes[37,61] and provides information on the formation of the isoxazolin-5-one heterocycle for the first time.[88]

4. Perspectives

4.1. Uptake of plant derived precursors

Upon comparison of the percentiles of amino acid incorporation into compounds **1** and **2** (see chapter 2.3.2.) with the specific amounts of available free amino acids in the host plant leafs[88], it becomes obvious that the ingestion of these nutrients by the leaf beetle larvae is not sufficient to explain the observed specific amounts of isoxazolin-5-one and 3-NPA moieties in these insects. This difference is evidence for the necessity of an uptake of further compounds deriving from the ingested and digested plant material. Firstly, such compounds could be intermediates on the biosynthetic pathway, starting from essential amino acids, especially valine, methionine as well as isoleucine, and finally yielding β-alanine, as the frontier between primary and secondary metabolism in leaf beetles in terms of compounds **1** and **2**. Secondly, a major nutrition source in Chrysomelina might be the digestion of proteins, resulting in the release of required amino acids. As an additional source of possible nutrients for the anabolism of compounds **1** and **2**, parallel pathways that provide β-alanine in leaf beetles, not yet validated, have to be taken into account, e.g. from spermine.[100]

To summarize these perspective results, anabolic biosynthetic pathways in general cannot be reduced to single substances as starting points that might be ingested from the food.

4.2. Enzymes involved in the biosynthesis of compounds 1 and 2

In first experiments on putative candidates for enzymes involved in the biosynthetic pathway of compounds **1** and **2** in juvenile Chrysomelina, synthetic [$^{13}C_6$]-**1** and [$^{13}C_6$]-**2** were used as standards for SIL-IS-quantifications within RNAi-approaches. At first, it was of interest to identify enzymes that catalyze the oxidation of the nitrogen atom of β-alanine. These reactions result in the formation of the oxime, and thus the heterocycle, and the actual toxin 3-NPA as well.[88] Thus, sequences from enzymes belonging to the cytochrome P450 family were identified in the beetles as putative candidates. After injection of corresponding P450-derived double-stranded RNA (dsRNA) into larval

hemolymph of *C. populi*, a decreased specific amount of compound **2** as well as a simultaneous increase in the amount of compound **1** could be detected (Fig. 38).

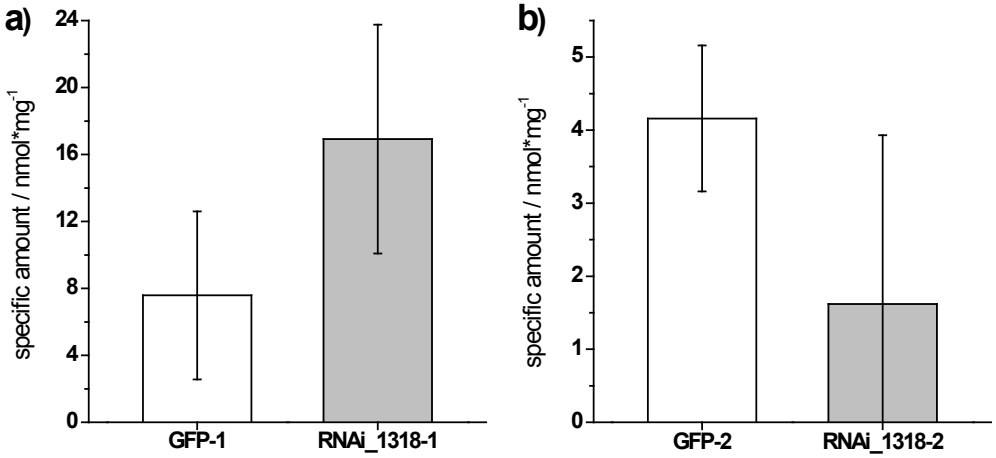


Figure 38 Specific amounts of compounds **1** (a) and **2** (b) after RNAi-treatments of *C. populi* larvae compared to GFP-controls; $N_{GFP} = 3$, $N_{RNAi} = 4$.

The dsRNA was provided by Toni Krause. In the future, compounds [$^{13}C_6$]-**1** and [$^{13}C_6$]-**2** will be used in the RNAi-based identification of further putative candidates for enzymes involved in the biosynthesis compounds **1** and **2** in Chrysomelina.

4.3. Biosynthetic pathways yielding compounds 1 and 2 in other organisms

Due to former experiments applying radioactive labelled precursors to unravel aspects of the biosynthesis of especially 3-NPA, e.g. in *I. spicata*, the picture of the formation of these compounds remains incomplete.[61] As shown within this thesis, it is not sufficient to rely only on radioactivity as a tracer for incorporation of biosynthetic intermediates.[88] As isoxazolin-5-one and 3-NPA moieties occur parallel in particular, as shown in *Astragalus* species,[7] it is likely that similar or equal metabolic pathways as described in this thesis for leaf beetles might also occur in plants and other organisms.

5. Zusammenfassung

5.1. Synthese von Isoxazolin-5-on-Glucosiden

Ein alternativer Zugang zu Isoxazolin-5-on-Glycosiden wurde realisiert, basierend auf einer Kaskadenreaktion.[74,75,77,80] Die Totalausbeute der Naturstoffsynthese von Verbindung **1** wurde von 1% (Koenigs-Knorr) auf 22% gesteigert.[4,77,80] In einem weiteren Reaktionsschritt wurde Verbindung **2** durch Transesterifizierung dargestellt (Fig. 39).[77]

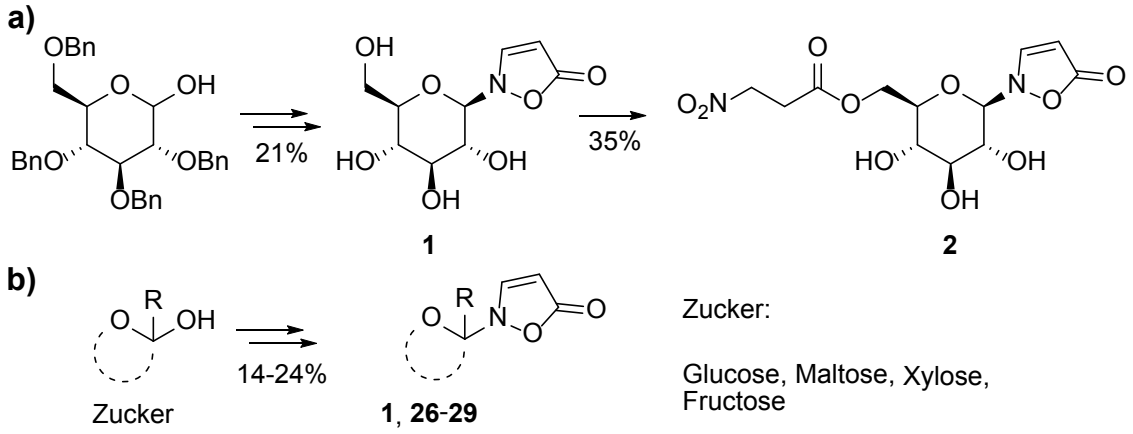


Figure 39 a) Neuer Ansatz zur Synthese der Verbindungen **1** und **2**, ausgehend von tetra-*O*-benzylglucose; b) Prinzip der Synthese von Isoxazolin-5-on-Glycosiden **1** und **26-29** ausgehend von ungeschützten Zuckern als Vorstufen.[77,88]

Die Anzahl von Synthesestufen wurde durch den Einsatz ungeschützter Zucker, die in die entsprechenden Oxime überführt wurden, weiter reduziert.[80] Unter Verwendung dieser Methode wurden Verbindung **1** sowie vier neue Isoxazolin-5-on-Glycoside, abgeleitet von Xylose, Maltose und Fructose synthetisiert, aufgereinigt und charakterisiert.[80] Kristalle von Verbindung **1** konnten isoliert werden, um eine Röntgenkristallstrukturanalyse durchzuführen. Alle Isoxazolin-5-on-Glycoside zeigten hohe Quantenausbeuten um 0,3 bei der photochemischen Zersetzung unter Einstrahlung von UV-Licht mit Wellenlängen von 254 nm im wässrigen Milieu und bei rt, analog zu ähnlichen Isoxazolin-5-on-Naturstoffen.[4,80] Diese Photoreaktionen resultierten in

quantitativer Freisetzung der korrespondierenden Zucker.[80] Verbindung **1** zeigt hohe Stabilität in Gegenwart von β-Glucosidase aus Mandeln.[80]

5.2. Ökologische Bedeutung von Isoxazolin-5-on-Derivaten in Blattkäfern

NMR- und HPLC-MS-basierte Analysen von Proben von juvenilen Chrysomelina Blattkäfern lieferten Evidenz für das Vorkommen von Isoxazolin-5-on- und 3-NPA-abgeleiteten Glucosiden, welche einzig von adulten Sekreten sowie Eiern dieser Spezies bekannt waren.[5,15] Nach Vergleich von Spektren der authentischen Synthesestandards dieser Isoxazolin-5-on-Glucoside mit Spektren der biologischen Proben wurde das Vorkommen von Verbindungen **1** und **2** in Chrysomelina Larven, den Puppen sowie der adulten Hämolymphe bestätigt.[87] Quantitative Analysen zeigten ökologisch signifikante spezifische Stoffmengen von Verbindungen **1** und **2** im Bereich einiger nmol/mg, was mit millimolaren Konzentrationen korreliert.[88] Die Verwendung des SIL-IS-Ansatzes reduziert signifikant die Analysenzeit, während die analytische Genauigkeit und Präzision optimiert werden.[89] Es wurde beobachtet, dass die spezifische Stoffmenge von Verbindung **2** in Chrysomelina Larven mit der Körpermasse ansteigt.[87,88] Die biologische Hauptfunktion von Verbindung **1** in Chrysomelina ist das Bereitstellen einer Vorstufe zur Veresterung mit dem Gift 3-NPA zur Bildung von Verbindung **2**, als Speichermolekül, das in der Insektenhämolymphe zirkuliert.[87] Diese Hypothese wird durch die hohe Konzentration von Verbindung **1** in der Hämolymphe gestützt, welche ausreichendes Transesterifizierungspotential garantiert. Außerdem wurde keinerlei Toxizität dieser Substanz beobachtet, nachdem signifikant konzentrierte Lösungen Zellkulturen von Pilzen, Bakterien sowie humanen Zelllinien zugesetzt wurden. Der beobachtete Entgiftungsweg in 3-NPA-Produzenten über eine Esterbildung wurde bis jetzt nicht beschrieben. Zudem erweitern diese Ergebnisse das Verständnis der chemischen Abwehr in juvenilen Chrysomelina Blattkäfern, durch die Produktion von Gift sowie aposematische Warnung über sekretierte Volatile, was zwei verschiedenen Verteidigungs-Modi zur selben Zeit entspricht.[24,25,87]

5.3. Biosynthese von Isoxazolin-5-on- und 3-NPA-Einheiten in Chrysomelina

Isotopenmarkierte und nicht-markierte putative Intermediate des Biosynthesewegs von Verbindungen **1** und **2** in Chrysomelina Larven wurden den Insekten oder Suspensionen ihres Fettkörpers zugesetzt. HPLC-MS sowie NMR-Analysen dieser Proben resultierten im Vorschlag für eine unerwartete metabolische Route zur Bildung von Verbindungen **1** und **2** in juvenilen Chrysomelina (Fig. 40).[37,61,88]

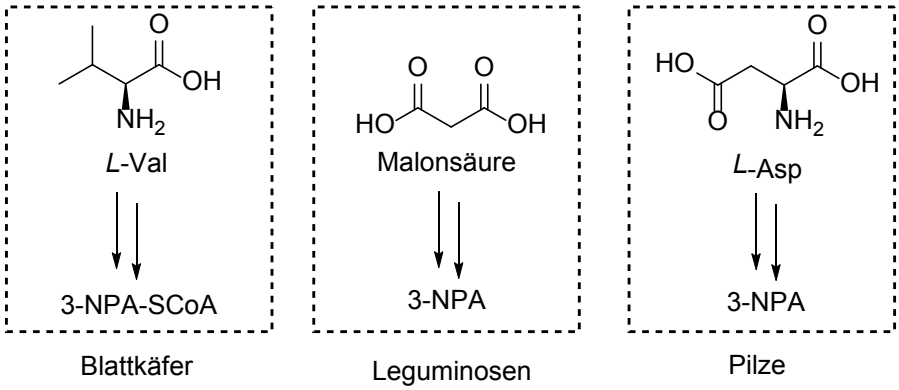


Figure 40 Vergleich verschiedener vorgeschlagener biosynthetischer Wege von 3-NPA.

Der neuartige vorgeschlagene Weg startet bei essentiellen Aminosäuren, z.B. Valin, und führt über Propanoyl-CoA und β-Alanin als Zwischenstufen. Weitere stufenweise Monooxygenierung am Stickstoffatom von β-Alanin führt zur Bildung der Intermediate 3-(Hydroxyamino)propansäure **30** sowie 3-(Hydroxyimino)propansäure **31**. Das Oxim **31** wird zyklisiert, um Isoxazolin-5-on **17** zu bilden, welches über α-UDP-Glucose in Verbindung **1** überführt wird. Weitere Oxidation des Oxims liefert 3-Nitropropansäure. Der korrespondierende CoA-Ester wird zuletzt in Verbindung **2** überführt. Quantitative Experimente belegten die Signifikanz der vorgeschlagenen Intermediate auf dem Biosyntheseweg. Diese Experimente zeigen, dass die Isoxazolin-5-on- sowie die 3-NPA-Einheiten in Chrysomelina Larven von den selben Vorstufen ausgehend gebildet werden. Malonsäure sowie Threonin zeigen keinen Einbau.[88] Eine direkte Decarboxylierung von Asparaginsäure zur Bildung von β-Alanin wurde nicht beobachtet.[6] Folglich ist die neue vorgeschlagene metabolische Route alternativ zu den vorher beschriebenen[37,61] und liefert zum ersten Mal Informationen zur Bildung des Isoxazolin-5-on-Heterozyklus.[88]

6. Ausblick

6.1. Aufnahme von Vorstufen aus den Pflanzen

Nach Vergleich des prozentualen Einbaus von Aminosäuren in die Verbindungen **1** und **2** (Vergleich Kapitel 2.3.2.) mit den spezifischen Stoffmengen verfügbarer freier Aminosäuren in den Blättern der Wirtspflanze[88] wird deutlich, dass die Aufnahme dieser Nährstoffe durch die Blattkäferlarve nicht ausreicht, um die beobachteten spezifischen Stoffmengen der Isoxazolin-5-on- und 3-NPA-Einheiten in diesen Insekten zu erklären. Diese Differenz ist Evidenz für die Notwendigkeit der Aufnahme weiterer Verbindungen, die sich vom aufgenommenen und verdauten Pflanzenmaterial ableiten. Erstens könnten solche Verbindungen Intermediaten des Biosynthesewegs entsprechen, wie zum Beispiel Valin, Methionin oder Isoleucin, die letztendlich in der Bildung von β-Alanin münden, der Grenzverbindung zwischen primärem und sekundärem Metabolismus in Blattkäfern in Bezug auf Verbindungen **1** und **2**. Zweitens stellt eine wesentliche Nährstoffquelle in Chrysomelina möglicherweise die Verdauung von Proteinen aus der aufgenommenen Wirtspflanze dar, die zur Freisetzung von Aminosäuren führt. Weiterhin könnten noch nicht validierte parallele Wege zur Bildung von β-Alanin in Chrysomelina führen, z.B. über Spermin.[100]

Um diese perspektivischen Resultate zusammen zu fassen lässt sich sagen, dass Biosynthesewege nicht auf einzelne Substanzen als Startpunkte reduziert werden können, die eventuell aus der Nahrung aufgenommen werden.

6.2. Enzyme in der Biosynthese von Verbindungen 1 und 2

In ersten Experimenten in Bezug auf putative Enzym-Kandidaten, die in die Biosynthese von Verbindungen **1** und **2** in juvenilen Chrysomelina involviert sind, wurden die synthetischen Verbindungen [$^{13}C_6$]-**1** und [$^{13}C_6$]-**2** als Standards für SIL-IS-Quantifizierungen in RNAi-Ansätzen verwendet. Zunächst war es von Interesse Enzyme zu identifizieren, welche die Oxidation des Stickstoffatoms in β-Alanin katalysieren. Diese Reaktionen resultieren in der Bildung des Oxims und somit des Heterozyklus sowie dem eigentlichen Giftstoff 3-NPA.[88] Deshalb wurden Sequenzen von Enzymen, die

zur Cytochrom P450-Familie gehören in den Käfern als putative Kandidaten identifiziert. Nach der Injektion von doppelsträngiger RNA (dsRNA), die zu P450-Sequenzen korrespondiert, in larvale Hämolymphe von *C. Populi*, wurde eine Abnahme der spezifischen Stoffmenge von Verbindung **2** sowie eine gleichzeitige Zunahme der Stoffmenge von Verbindung **1** detektiert (Fig. 41).

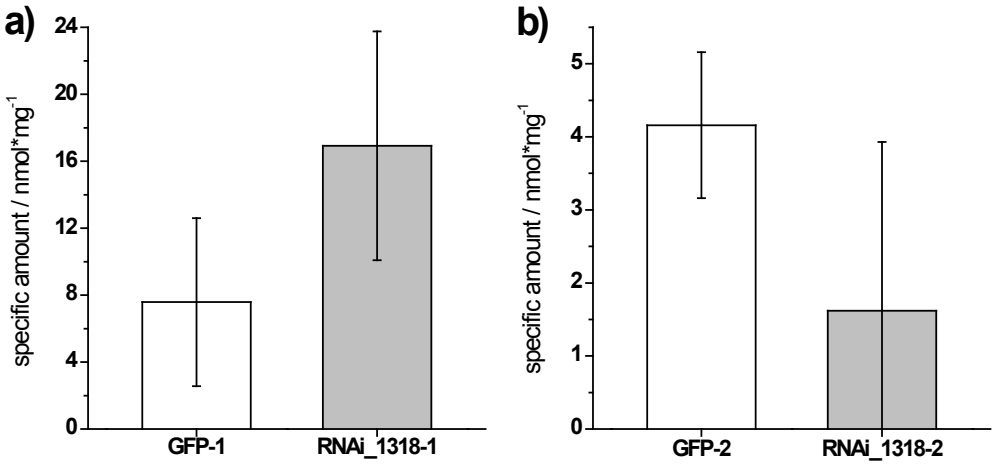


Figure 41 Spezifische Stoffmengen von Verbindung **1** (a) und **2** (b) nach RNAi-Behandlung von *C. populi* Larven im Vergleich zu GFP-Kontrollen. N_{GFP}=3, N_{RNAi}=4.

Die dsRNA wurde von Toni Krause bereitgestellt. In zukünftigen Experimenten sollen [$^{13}C_6$]-**1** und [$^{13}C_6$]-**2** in RNAi-basierten Identifikationen weiterer putativer Kandidaten von Enzymen auf dem Biosyntheseweg von Verbindungen **1** und **2** verwendet werden.

6.3. Biosynthesewege von Verbindungen 1 und 2 in anderen Organismen

Aufgrund von vorherigen Versuchen auf der Basis von radioaktiv markierten Vorstufen, die verwendet wurden, um Aspekte der Biosynthese, z.B. von 3-NPA in *I. spicata*, aufzuklären, bleibt das Gesamtbild der Entstehung dieser Verbindungen unvollständig.[61] Wie in dieser Arbeit gezeigt wurde ist es nicht ausreichend, sich einzig auf Radioaktivität als Marker für biosynthetische Vorstufen zu verlassen.[88] Da Isoxazolin-5-on- und 3-NPA-Einheiten in einigen Organsimen gleichzeitig auftreten, wie in *Astragalus* Spezies gezeigt wurde,[7] ist es naheliegend, dass die Biosynthesen dieser Verbindungen in Pflanzen und anderen Organismen ähnlich oder identisch zu den in Blattkäfern gefundenen sind.

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8. Original Manuscripts and Publication Equivalents

8.1. General remarks

In this section the original publications that form the basis of this thesis are shown. For this purpose, reprint permissions for every publication were obtained from the publishers. The electronic supporting information (ESI) of each publication is provided. Unpublished results are discussed in the chapter "General Discussion" together with the findings published in the articles that are mentioned as follows.

8.2. Manuscript 1

Publikation: Becker, T.[1], Görls, H.[2], Pauls, G.[3], Wedekind, R.[4], Kai, M.[5], von Reuß, S. H.[6], Boland, W.[7] (2013). Synthesis of Isoxazolin-5-one glucosides by a cascade reaction. The Journal of Organic Chemistry, 78, 12779-12783. DOI: 10.1021/jo4023155.
Wird diskutiert in den Kapiteln: 2.1.1., 3.1. und 5.1.

Beteiligt an	**Autor Nummer**						
	1	2	3	4	5	6	7
Konzeption	X					X	X
Planung	X						X
Datenerhebung	X	X	X	X	X		
Datenanalyse und -interpretation	X				X	X	X
Schreiben des Manuskripts	X					X	X
Publikationsäquivalente	1.0						

The subsequent manuscript has been used in the PhD thesis of Gerhard Pauls to show how compounds **1** and **2** have been synthesized to use them for quantification of these substances in natural samples. The properties, especially in terms of HPLC-MS separation, have been discussed.

JOC The Journal of Organic Chemistry

Article

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Synthesis of Isoxazolin-5-one Glucosides by a Cascade Reaction

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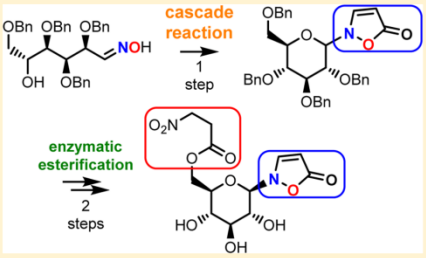
Supporting Information

ABSTRACT: A novel synthetic route was developed for the construction of isoxazolin-5-one glucosides using a cascade reaction. An X-ray crystal structure analysis of a isoxazolin-5-one glucoside confirmed the structure and stereochemistry of the heterocycle. The properties of the α- and β-anomers of the isoxazolin-5-one glucosides were compared. The first synthesis of 2-[6′-(3″-nitropropanoyl)-β-D-glucopyranosyl]-3-isoxazolin-5-one was realized by direct enzymatic esterification without need of further protective groups.



INTRODUCTION

Isoxazolin-5-one glucosides are natural compounds that were identified as metabolites in legumes (Fabaceae) as well as in certain species of leaf beetles (Chrysomelidae).[1−6] 2-(β-D-Glucopyranosyl)-3-isoxazolin-5-one (**1**) and its nitropropanoyl derivative **2** have been reported to be major components of the defensive secretions of adult leaf beetles.[1−3] Seedlings of *Lathyrus odoratus* show concentrations of compound **1** of up to 0.8% of their dry mass (figure 1).[4]

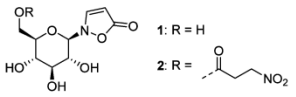


Figure 1. Isoxazolin-5-one glucosides.

A variety of different glucose derivatives of 3-nitropropanoic acid (3-NPA) have been found in several plant, insect, and fungi families.[7] It has been shown that the acid component 3-NPA, is an irreversible inhibitor of succinate dehydrogenase.[8] As a consequence the mitochondrial citric acid cycle is inhibited, causing neurodegenerative symptoms.[9] The esters of 3-NPA serve as pretoxins and storage molecules in different organisms and represent an important class of defensive compounds.[10−13] In order to provide standards for studies concerning the biosynthesis, quantification, and hydrolysis kinetics of **2,** the synthesis of compounds **1** and **2** is of interest. In the case of compound **1**, no efficient synthesis is known, and no synthesis of the 3-NPA derivative **2** has been reported.

The regioselective synthesis of pyranose esters via transesterification has been intensively studied.[14−18] Different strategies for the construction of isoxazolin-5-one moieties have been reported.[19−24] Van Rompuy et al. described a nucleophilic substitution of organohalides with the sodium salt of isoxazolin-5-one.[20−22] This approach requires complicated purification procedures and provides low yields because of the formation of many side products when applied to the synthesis of glucose derivatives.[22] Hence, Baldwin and co-workers developed an alternative method based on a 5-endo-dig cyclization reaction to synthesize amino acid derivatives of isoxazolin-5-one.[23−26]

Herein we report a novel direct synthetic route for compound **1** based on a cascade reaction consisting of a 6-exo-trig ring closure followed by a 5-endo-dig reaction. The synthesis of 2-[6′-(3″-nitropropanoyl)-β-D-glucopyranosyl]-3-isoxazolin-5-one (**2**) was achieved by the regioselective transesterification of an activated ester of 3-NPA to compound **1**. Furthermore our novel synthetic strategy allows for the first time the synthesis of α-configured 3,4-unsaturated isoxazolin-5-one glucosides.

RESULTS AND DISCUSSION

We commenced our work with the synthesis of (*E*)- and (*Z*)-2,3,4,6-tetra-*O*-benzyl-D-glucose oxime (**3**) starting from commercially available 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose (TOBG).[27] An excess of hydroxylamine formed in situ via deprotonation of its hydrochloride salt by sodium ethanolate solution in ethanol[28] afforded isomers (*E*/*Z*)-**3** in quantitative yield (Scheme 1).

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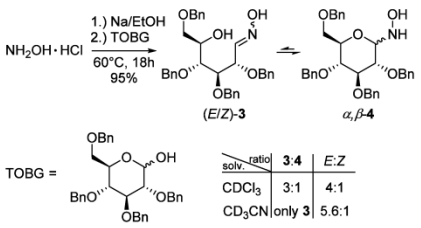
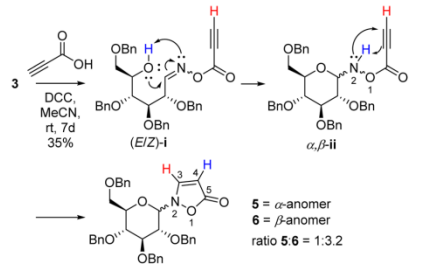
Scheme 1. Solvent-Dependent Formation of 3 and 4



A solvent-dependent equilibrium between the aldoxime forms (*E*/*Z*)-**3** and the *N*-hydroxylamine pyranose isomers α,β-**4** was observed. ^{1}H NMR spectra in $CDCl_3$ showed a relative **3**/**4** ratio of 3:1, while in CD_3CN the cyclic isomers α,β-**4** were absent. Because of the complexity of the mixture, the α/β-ratio of isomers **4** was not determined.

We found (*E*/*Z*)-**3** to be transformed into isoxazolin-5-one derivatives **5** and **6** in a one-pot synthesis when propynoic acid was applied under Steglich conditions[29] in MeCN (Scheme 2).

Scheme 2. Proposed Mechanism and Reaction Pathway for the Cascade Reaction



The reaction is initiated by a chemoselective acylation of aldoxime **3** with propynoic acid to produce intermediates (*E*/*Z*)-**i** followed by two consecutive ring-closure steps to provide a mixture of **5** and **6**. The course of the reaction was followed by ^{1}H and ^{13}C NMR measurements at rt, which provided evidence for the formation of intermediates (*E*/*Z*)-**i** by a low-field shift of the oxime doublets from 7.41 ppm (H_E-1) and 6.87 ppm (H_Z-1) to 7.95 and 7.85 ppm, respectively. We observed that the H-1 oxime signals in **3** disappeared nearly quantitatively after the application of DCC/propynoic acid. Over a period of several days the integrals of the oxime doublets decreased while the intensities of the isoxazolin-5-one doublets increased. This observation can be explained by an intramolecular nucleophilic attack of the 5′-OH group on the 1′-carbon of the oxime moiety in (*E*/*Z*)-**i** (6-exo-trig reaction) to form the α-and β-anomers (α,β-**ii**). The isomers α,β-**ii** are transformed into the corresponding isoxazolin-5-one glucosides by a second nucleophilic attack of the nitrogen atom on the β-position of the propynoyl group (5-endo-dig reaction). For these sequential nucleophilic processes, the terms nucleophilic cascade reaction[30] or homodomino reaction[31] can be used. The integration of the heterocyclic protons (H-3 and H-4) in the ^{1}H NMR spectra indicated that the α- and β-anomers **5** and **6** are formed in a ratio of ca. 1:3. Compounds **5** and **6** were isolated by column chromatography, and their structures were confirmed by NMR, IR, and UV spectroscopy as well as HRMS. In addition, an X-ray crystal structure analysis of compound **6** was performed after crystallization from ethanol/ethyl acetate (Figure 2); this represents the first crystal

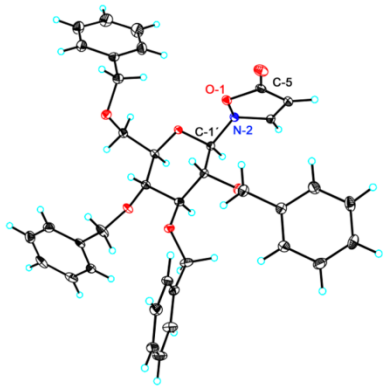


Figure 2. Ellipsoid plot of glucoside **6** (50% probability). Gray, carbon; red, oxygen; blue, nitrogen; green, hydrogen. Hydrogen atoms have been drawn with arbitrary units.

structure of an isoxazolin-5-one glycoside. The resulting bond lengths and angles confirmed the structure of the heterocycle as an isoxazolin-5-one instead of its isoxazolin-3-one isomer, as shown. This observation rules out a possible intramolecular propynoyl transfer from O-1 to N-2 in **ii**. Furthermore, the absolute configuration of compound **6** was confirmed.

The ^{1}H NMR spectra of compounds **5** and **6** show doublet signals with chemical shifts and coupling constants typical for isoxazolin-5-one moieties (8.3−8.1 and 5.3−5.1 ppm; $J_{3,4}$ = 3.7 Hz)[21,23,24] and anomeric protons of the glucose ring (α: 5.53 ppm, $J_{1',2'}$ = 5.7 Hz and β: 4.90 ppm, $J_{1',2'}$ = 9.1 Hz). The ^{13}C NMR spectra show signals at 154.8 (C_α-3), 155.2 (C_β-3), 89.3 (C_α-4), 92.8 (C_β-4), 85.9 (C_α-1′), and 89.2 ppm (C_β-1′). The glucosides **5** and **6** show UV absorptions around 205 nm (ε = 39 000 L mol^{-1} cm^{-1}) and 260 nm (ε = 12 100 L mol^{-1} cm^{-1}) corresponding to the benzyl and isoxazolin-5-one moieties. The IR spectra show two characteristic absorptions at 1750 cm^{-1} (C═O stretch) and 1550 cm^{-1} (C═C stretch of the isoxazolin-5-one double bond). The spectroscopic data are consistent with literature values.[21−24]

Deprotection of compounds **5** and **6** was achieved using boron trichloride at −84 °C in dichloromethane[32] to give 2-(α-D-glucopyranosyl)-3-isoxazolin-5-one (**8**) and 2-(β-D-glucopyranosyl)-3-isoxazolin-5-one (**1**) in high yields and purity (Scheme 3). The spectral data for compound **1** are identical with literature values.[3,22] The ^{1}H NMR spectrum of compound **8** shows doublets at 8.52 and 5.39 ppm ($J_{3,4}$ = 3.7 Hz) corresponding to the isoxazolin-5-one moiety, which can be discriminated from those of the β-anomer **1** (8.49 and 5.52 ppm, $J_{3,4}$ = 3.7 Hz). The anomeric proton in compound **8** (H_α-1′) shows a doublet at 5.73 ppm ($J_{1',2'}$ = 6.1 Hz), while the H_β-1′ doublet shows a signal at 5.14 ppm ($J_{1',2'}$ = 9.2 Hz). The

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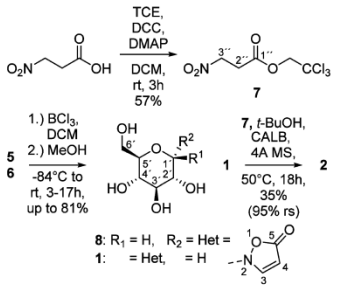
Scheme 3. Reaction Conditions for the Synthesis of Compounds 1, 2, 7, and 8



corresponding ^{13}C NMR signals appear at 154.8 (C_{α}-3), 155.2 (C_{β} −3), 88.2 (C_{α}-4), 92.8 (C_{β}-4), 87.1 (C_{α}-1′), and 89.2 ppm (C_{β}-1′). NMR spectra showed that compounds **1** and **8** are stable in D_2O and CD_3OD at rt and neutral pH. The glucosides **1** and **8** exhibit strong UV absorption at 261 nm (ε = 10 800 L mol^{-1} cm^{-1}) as well as IR absorptions typical for isoxazolin-5-one moieties at 1720−1730 cm^{-1} (C═O stretch) and 1550 cm^{-1} (C═C stretch).

The regioselective acylation of glucosides can be achieved without the use of protective groups by enzymatic transesterification reactions in organic solvents.[14] 2,2,2-Trichloroethyl 3-nitropropanoate (**7**) was synthesized applying Steglich conditions[29] to commercially available 3-NPA and 2,2,2-trichloroethanol (TCE) and purified by chromatography. Esterification of **1** was realized using immobilized *Candida antarctica* lipase B (CALB) and **7** as an activated acyl transfer agent to obtain 2-[6′-(3″-nitropropanoyl)-β-D-glucopyranosyl]-3-isoxazolin-5-one (**2**) (Scheme 3). A regioselectivity of about 95% was determined. The nonconverted 2-(β-D-glucopyranosyl)-3-isoxazolin-5-one **1** could be recovered by chromatography. The spectral data of compound **2** were identical with the literature values.[3]

■ CONCLUSION

In conclusion, the first total synthesis of naturally occurring 2-[6′-(3″-nitropropanoyl)-β-D-glucopyranosyl]-3-isoxazolin-5-one (**2**) using an efficient four-step synthetic route is reported. A novel cascade reaction pathway was found to incorporate 3,4-unsubstituted isoxazolin-5-one moieties at the 1-position of glucose to afford 2-(β-D-glucopyranosyl)-3-isoxazolin-5-one (**1**). In addition, the first synthesis of α-configured isoxazolin-5-one glucosides (**5** and **8**) has been described. Finally, we have presented the first crystal structure of an isoxazoline-5-one glycoside.

■ EXPERIMENTAL SECTION

General Experimental Methods. Melting points were determined using a capillary melting point apparatus. Infrared spectra were measured with a IR spectrometer over the 700−4000 cm^{-1} range in transmission mode with a spectral resolution of 2 cm^{-1}. Optical rotations were measured at 589 nm and 22 °C. Ultraviolet spectra were recorded on a UV spectrophotometer over the range from 190 to 300 nm. NMR spectra were measured using a spectrometer operating at 500 MHz (^1H) and 125 MHz (^{13}C). Chemical shifts (δ) are quoted in parts per million (ppm) and are referenced to the signals of residual protonated solvents (CD_2HCN at 1.94 ppm and $CHCl_3$ at 7.26 ppm). Methanol was added as a reference for ^{13}C NMR spectra in D_2O. Assignment of peaks was carried out using 2D NMR experiments (COSY, HSQC, and HMBC). The multiplicities are given as follows: br, broad; s, singlet; d, doublet; t, triplet; dd, doublet of doublets; ddd, doublet of doublets of doublets; m, multiplet. High-resolution mass spectra were recorded on a UHR-qTOF and an APCI-OrbitrapXL mass spectrometer. The intensity data for the X-ray analysis were collected on a diffractometer using graphite-monochromatized Mo Kα radiation. Data were corrected for Lorentz and polarization effects but not for absorption effects.[33,34] The structures were solved by direct methods (SHELXS[35]) and refined by full-matrix least-squares techniques against F_o^2 (SHELXL-97[35]). HPLC-MS analyses were carried out using the APCI mode (vaporizer temperature 500 °C, capillary temperature 300 °C) connected to an HPLC system equipped with an RP18 column. Thin-layer chromatography was performed on TLC silica gel 60 F_{254} aluminum sheets. Compounds containing nitro groups were visualized using the modified Griess assay. Preparative column chromatography was carried out using silica gel (30−60 μm). All chemicals were purchased in the highest purity that was commercially available and used without further purification. All solvents except for diethyl ether were purchased in HPLC grade and used without further purification. Dichloromethane, *tert*-butyl alcohol, and acetonitrile were dried over activated molecular sieves (4 Å) under an argon atmosphere. Diethyl ether was distilled before use.

Synthesis of 2,3,4,6-Tetra-*O*-benzyl-2-(α-D-glucopyranosyl)-3-isoxazolin-5-one (5) and 2,3,4,6-Tetra-*O*-benzyl-2-(β-D-glucopyranosyl)-3-isoxazolin-5-one (6). To a solution of propynoic acid (1.12 g, 16 mmol, 1.1 equiv) in 1 mL of dry MeCN, a solution of oxime **3** (8.0 g, 14.4 mmol) in 7 mL and a solution of DCC (3.09 g, 15 mmol, 1.04 equiv) in 5.3 mL of dry MeCN were added dropwise over 20 min simultaneously at rt. After 7 days of stirring at rt, the mixture was concentrated under reduced pressure at 30 °C, and the residue was taken up in diethyl ether (10 mL). The colorless precipitate was filtered off, and the filtrate was concentrated under reduced pressure at 30 °C. The crude product was purified by column chromatography (DCM/MeCN 100:1 and $CHCl_3$/ethyl acetate 95:5). Methanol was added, and the solvents were removed under reduced pressure. This procedure was repeated five times to yield the title compound **6** as a colorless solid (2.5 g, 4.11 mmol, 28.5%). Crystals suitable for an X-ray crystal structure analysis were obtained via recrystallization from ethanol/ethyl acetate. $[\alpha]_D^{22}$ −16.6 (*c* 0.55, $CHCl_3$); R_f = 0.30 (ethyl acetate/$CHCl_3$ 5:95); ^1H NMR (500 MHz, CD_3CN) δ 8.16 (d, $J_{3,4}$ = 3.7 Hz, 1H, H-3), 7.36−7.20 (m, 20H, Ar−H), 5.33 (d, $J_{3,4}$ = 3.7 Hz, 1H, H-4), 4.90 (d, $J_{1',2'}$ = 9.1 Hz, 1H, H-1′), 4.87 (d, *J* = 11.2 Hz, 1H, OCH_2Ph), 4.84 (d, *J* = 11.2 Hz, 1H, OCH_2Ph), 4.78 (d, *J* = 10.9 Hz, 1H, OCH_2Ph), 4.76 (d, *J* = 11.0 Hz, 1H, OCH_2Ph), 4.63 (d, *J* = 11.0 Hz, 1H, OCH_2Ph), 4.57 (d, *J* = 10.9 Hz, 1H, OCH_2Ph), 4.52 (d, *J* = 12.1 Hz, 1H, OCH_2Ph), 4.48 (d, *J* = 12.1 Hz, 1H, OCH_2Ph), 3.90 (t, $J_{1',2'}$ = 9.1 Hz, 1H, H-2′), 3.76−3.72 (m, 1H, H-3′), 3.68−3.62 (m, 2H, H-6′), 3.61−3.56 (m, 2H, H-4′ and H-5′); ^{13}C NMR (125 MHz, CD_3CN) δ 171.6 (C-5), 155.2 (C-3), 139.6 (Ar-Cq), 139.3 (Ar-Cq), 139.3 (Ar-Cq), 139.0 (Ar-Cq), 129.4 (Ar-C), 129.4 (Ar-C), 129.3 (Ar-C), 129.3 (Ar-C), 129.2 (Ar-C), 129.2 (Ar-C), 129.1 (Ar-C), 129.0 (Ar-C), 128.9 (Ar-C), 128.8 (Ar-C), 128.8 (Ar-C), 128.8 (Ar-C), 128.7 (Ar-C), 128.6 (Ar-C), 128.6 (Ar-C), 128.5 (Ar-C), 92.8 (C-4), 89.2 (C-1′), 86.0 (C-3′), 78.9 (C-2′), 78.3 (C-4′ or C-5′), 77.7 (C-4′ or C-5′), 76.2 (OCH_2Ph), 75.6 (OCH_2Ph), 75.4 (OCH_2Ph), 73.7 (OCH_2Ph), 69.4 (C-6′); HRMS (APCI-Orbitrap) *m*/*z* calcd for $C_{37}H_{37}NO_7Na$ 630.2462 [M + Na]$^+$, found 630.2449; IR (thin film, cm^{-1}) 3063 (m), 3031 (m), 2961 (m), 2915 (m), 2869 (m), 1750 (s), 1556 (m), 1090 (s); UV (MeOH) λ_{max}/nm (ε/L mol^{-1} cm^{-1}) 205 (39340 ± 650), 261 (12140 ± 200); mp 97−99 °C. Compound **6** crystallized with two symmetrically independent molecules per asymmetric unit. The two molecules were identical except for the orientation of one benzyl group. The hydrogen atoms of the isoxazolin-5-one ring were located by difference Fourier synthesis and refined isotropically. All other hydrogen atoms were included at calculated positions with fixed thermal parameters. All non-hydrogen atoms were refined anisotropically.[35] XP was used for structure representations. Crystal data for **6**: $C_{37}H_{37}NO_7$, M_r = 607.703 g mol^{-1},

colorless prism, size 0.10 mm × 0.10 mm × 0.09 mm, triclinic, space group $P1$, a = 10.6443(5) Å, b = 12.4845(6) Å, c = 12.8966(6) Å, α = 104.609(2)°, β = 96.556(2)°, γ = 104.520(2)°, V = 1576.38(13) Å^3, T = 23 °C, Z = 2, ρ_{calcd} = 1.280 g cm^{-3}, μ(Mo Kα) = 0.88 cm^{-1}, F(000) = 644, 70 532 reflections in h(−12/12), k(−14/14), l(−15/15) measured in the range 2.72° ≤ Θ ≤ 25.03°, completeness Θ_{max} = 99.9%, 11066 independent reflections, R_{int} = 0.0209, 10606 reflections with $F_o > 4\sigma(F_o)$, 1107 parameters, 3 restraints, R_1(obs) = 0.0253, wR_2(obs) = 0.0614, R_1(all) = 0.0273, wR_2(all) = 0.0626, GOF = 1.033, Flack parameter = 0.0(3), largest difference peak/hole 0.161/−0.155 e Å^{-3}. Crystallographic data (excluding structure factors) have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication CCDC-961971. Copies of the data can be obtained free of charge from the CCDC, 12 Union Road, Cambridge CB2 1EZ, U.K. (E-mail: deposit@ccdc.cam.ac.uk).

The α-anomer **5** was isolated as a colorless oil (605 mg, 0.99 mmol, 6.8%). $[\alpha]_D^{22}$ +78.5 (*c* 0.51, $CHCl_3$); R_f = 0.41 (ethyl acetate/$CHCl_3$ 5:95); ^{1}H NMR (500 MHz, CD_3CN) δ 8.26 (d, $J_{3,4}$ = 3.7 Hz, 1H, H-3), 7.36−7.21 (m, 20H, Ar−H), 5.53 (d, $J_{1',2'}$ = 5.7 Hz, 1H, H-1′), 5.17 (d, $J_{3,4}$ = 3.7 Hz, 1H, H-4), 4.88 (d, *J* = 11.1 Hz, 1H, OCH_2Ph), 4.80 (d, *J* = 11.1 Hz, 2H, OCH_2Ph) 4.65 (s, 2H, OCH_2Ph), 4.57 (d, *J* = 11.0 Hz, 1H, OCH_2Ph), 4.51 (d, *J* = 11.9 Hz, 1H, OCH_2Ph), 4.46 (d, *J* = 11.9 Hz, 1H, OCH_2Ph), 4.10 (t, $J_{3',4'}$ = 9.1 Hz, 1H, H-3′), 3.95 (dd, $J_{2',3'}$ = 9.6 Hz, $J_{1',2'}$ = 5.8 Hz, 1H, H-2′), 3.89−3.85 (m, 1H, H-5′), 3.68−3.62 (m, 2H, H-6′), 3.60 (dd, $J_{4',5'}$ = 10.2 Hz, $J_{3',4'}$ = 8.7 Hz, 1H, H-4′); ^{13}C NMR (125 MHz, CD_3CN) δ 171.7 (C-5), 154.8 (C-3), 139.7 (Ar-Cq), 139.4 (Ar-Cq), 139.3 (Ar-Cq), 138.7 (Ar-Cq), 129.4 (Ar-C), 129.3 (Ar-C), 129.2 (Ar-C), 129.1 (Ar-C), 128.9 (Ar-C), 128.9 (Ar-C), 128.8 (Ar-C), 128.6 (Ar-C), 128.5 (Ar-C), 89.3 (C-4), 85.9 (C-1′), 82.8 (C-3′), 79.7 (C-2′), 78.2 (C-4′), 75.9 (OCH_2Ph), 75.5 (OCH_2Ph), 75.4 (C-5′), 74.2 (OCH_2Ph), 73.8 (OCH_2Ph), 69.7 (C-6′); HRMS (APCI-Orbitrap) *m*/*z* calcd for $C_{37}H_{41}N_2O_7$ 625.2908 $[M + NH_4]^+$, found 625.2892; IR (thin film, cm^{-1}) 3087 (m), 3062 (m), 3030 (m), 2923 (m), 2867 (m), 1749 (s), 1552 (m), 1093 (s); UV (MeOH) λ_{max}/nm (ε/L mol^{-1} cm^{-1}) 204 (36740 ± 600), 264 (12110 ± 200).

Synthesis of 2,2,2-Trichloroethyl 3-Nitropropanoate (7). 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 ($CHCl_3$) and removal of the solvent at 40 °C under reduced pressure, a colorless powder of **7** (834 mg, 3.33 mmol, 57.7%) was obtained. R_f = 0.78 ($CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.81 (s, 2H, CH_2CCl_3), 4.72 (t, $J_{2,3}$ = 6.1 Hz, 2H, CH_2NO_2), 3.16 (t, $J_{2,3}$ = 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; IR (thin film, cm^{-1}) 3012 (w), 2961 (m), 2926 (m), 1747 (s), 1549 (s), 1088 (s); mp 35−36 °C.

Synthesis of 2-(β-D-Glucopyranosyl)-3-isoxazolin-5-one (1). 2,3,4,6-Tetra-*O*-benzyl-2-(β-D-glucopyranosyl)-3-isoxazolin-5-one (**6**) (1.73 g, 2.85 mmol) was dissolved in dry DCM (150 mL) and cooled to −84 °C under an argon atmosphere. Boron trichloride (20 mL, 1 M, heptane, 7 equiv) was added dropwise. After 17 h at −84 to −79 °C, the mixture was quenched with methanol (20 mL) and warmed to rt. The solvents were removed at 25 °C under reduced pressure to yield a colorless oil. The residue was dissolved in methanol (5 mL), and silica (2 g) was added. The solvent was removed, and the residual colorless powder was applied to a silica gel column. After elution of the column (DCM/MeOH 5:1 to 2:1), the pure fractions were concentrated under high vacuum at rt to yield **1** as a colorless solid (522 mg, 2.11 mmol, 74.2%). $[\alpha]_D^{22}$ +13.5 (*c* 0.53, MeOH); R_f = 0.49 (DCM/MeOH 2:1); ^{1}H NMR (500 MHz, D_2O) δ 8.49 (d, $J_{3,4}$ = 3.7 Hz, 1H, H-3), 5.50 (d, $J_{3,4}$ = 3.7 Hz, 1H, H-4), 5.14 (d, $J_{1',2'}$ = 9.2 Hz, 1H, H-1′), 3.92−3.88 (m, 2H, H_A-6′ and H-2′), 3.73 (dd, J_1 = 6.9 Hz, J_2 = 5.6 Hz, 1H, H_B-6′), 3.64−3.57 (m, 2H, H-3′ and H-5′), 3.49 (t, $J_{3',4'}$ = 9.5 Hz, 1H, H-4′); ^{13}C NMR (125 MHz, D_2O) δ 174.8 (C-5), 154.8 (C-3), 91.1 (C-4), 88.8 (C-1′), 78.8 (C-5′), 76.7 (C-3′), 70.0 (C-2′), 69.5 (C-4′), 61.0 (C-6′); HRMS (ESI-TOF) *m*/*z* calcd for $C_9H_{13}NO_7Na$ 270.0584 $[M + Na]^+$, found 270.0575; IR (thin film, cm^{-1}) 3370 (br, s), 2932 (m), 1725 (s), 1544 (s), 1104 (s), 1040 (s); UV (MeOH) λ_{max}/nm (ε/L mol^{-1} cm^{-1}) 262 (10800 ± 200). The spectral data were in agreement with the literature values.[3,22]

Synthesis of 2-(α-D-Glucopyranosyl)-3-isoxazolin-5-one (8). 2,3,4,6-Tetra-*O*-benzyl-2-(α-D-glucopyranosyl)-3-isoxazolin-5-one (**5**) (100 mg, 0.165 mmol) was dissolved in dry DCM (8 mL) and cooled to −84 °C under an argon atmosphere. Boron trichloride (2 mL, 1 M, heptane, 10 equiv) was added dropwise. After 1.5 h at −84 to −79 °C, the mixture was warmed to rt and stirred for 1.5 h. The reaction was quenched with methanol (4 mL) at −75 °C and stirred for 30 min. The solvents were removed at 25 °C under reduced pressure to yield a colorless oil. The residue was dissolved in methanol (5 mL), and silica (500 mg) was added. The solvent was removed, and the residual colorless powder was applied to a silica gel column. After elution of the column (DCM/MeOH 5:1 to 2:1), the product fractions were concentrated under high vacuum at rt to yield **8** as a colorless oil (33.3 mg, 0.135 mmol, 81.8%). $[\alpha]_D^{22}$ +85.3 (*c* 1.5, MeOH); R_f = 0.49 (DCM/MeOH 2:1); ^{1}H NMR (500 MHz, D_2O) δ 8.52 (d, $J_{3,4}$ = 3.6 Hz, 1H, H-3), 5.73 (d, $J_{1',2'}$ = 6.1 Hz, 1H, H-1′), 5.39 (d, $J_{3,4}$ = 3.6 Hz, 1H, H-4), 4.10 (t, $J_{2',3'}$ = 9.6 Hz, 1H, H-3′), 4.02 (dd, $J_{2',3'}$ = 10.0 Hz, $J_{1',2'}$ = 6.2 Hz, 1H, H-2′), 3.87−3.80 (m, 2H, H-5′ and H_A-6′), 3.78−3.73 (m, 1H, H_B-6′) 3.53 (t, $J_{3',4'}$ = 9.5 Hz, 1H, H-4′); ^{13}C NMR (125 MHz, D_2O) δ 174.9 (C-5), 154.8 (C-3), 88.2 (C-4), 87.1 (C-1′), 77.1 (C-5′), 74.1 (C-3′), 70.6 (C-2′), 69.7 (C-4′), 61.1 (C-6′); HRMS (APCI-Orbitrap) *m*/*z* calcd for $C_9H_{14}NO_7$ 248.0765 $[M + H]^+$, found 248.0762; IR (thin film, cm^{-1}) 3382 (br, s), 2962 (m), 2923 (m), 1727 (s), 1552 (s), 1192 (s), 1063 (s); UV (MeOH) λ_{max}/nm (ε/L mol^{-1} cm^{-1}) 261 (10760 ± 200).

Synthesis of 2-[6′-(3″-Nitropropanoyl)-β-D-glucopyranosyl]-3-isoxazolin-5-one (2). A mixture of 2-(β-D-glucopyranosyl)-3-isoxazolin-5-one (**1**) (100 mg, 0.404 mmol), 2,2,2-trichloroethyl 3-nitropropanoate (**7**) (160.1 mg, 0.639 mmol), immobilized *C. antarctica* lipase B (150 mg), and 4 Å molecular sieves was suspended in dry *tert*-butyl alcohol (7 mL). The suspension was stirred at 50 °C under an argon atmosphere for 18 h. The enzyme was filtered off, and the filter cake was washed with *tert*-butyl alcohol (2 × 5 mL) at 40 °C and cold methanol (5 mL). The filtrate was concentrated under reduced pressure at 25 °C, and the residue was taken up in methanol. Silica was added, and the solvent was evaporated under reduced pressure at 25 °C to obtain a colorless powder. The dry powder 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 (50 mg, 0.144 mmol, 35.6%). Nonconverted glucoside **1** could be recovered (31 mg, 0.125 mmol, 31%). $[\alpha]_D^{22}$ +30.1 (*c* 0.36, MeOH); R_f = 0.20 (ethyl acetate/MeOH/DCM 10:1:1); ^{1}H NMR (500 MHz, D_2O) δ 8.47 (d, $J_{3,4}$ = 3.7 Hz, 1H, H-3), 5.52 (d, $J_{3,4}$ = 3.7 Hz, 1H, H-4), 5.15 (d, $J_{1',2'}$ = 9.2 Hz, 1H, H-1′), 4.82 (t, $J_{2'',3''}$ = 5.8 Hz, 2H, H-3″), 4.51 (dd, $J_{A6',B6'}$ = 12.3 Hz, $J_{5',A6'}$ = 2.2 Hz, 1H, H_A-6′), 4.33 (dd, $J_{A6',B6'}$ = 12.4 Hz, $J_{5',B6'}$ = 5.2 Hz, 1H, H_B-6′), 3.91 (t, $J_{1',2'}$ = 9.2 Hz, 1H, H-2′), 3.78 (ddd, $J_{4',5'}$ = 10.0 Hz, $J_{5',B6'}$ = 5.2 Hz, $J_{5',A6'}$ = 2.2 Hz, 1H, H-5′), 3.62 (t, $J_{2',3'}$ = 9.3 Hz, 1H, H-3′), 3.50 (t, $J_{3',4'}$ = 9.5 Hz, 1H, H-4′), 3.13 (t, $J_{2'',3''}$ = 5.8 Hz, 2H, H-2″); ^{13}C NMR (125 MHz, D_2O) δ 174.7 (C-5), 172.4 (C-1″), 155.0 (C-3), 91.7 (C-4), 88.7 (C-1′), 76.5 (C-5′), 76.1 (C-3′), 70.7 (C-3″), 69.9 (C-2′), 69.5 (C-4′), 63.9 (C-6′), 31.6 (C-2″); HRMS (ESI-TOF) *m*/*z* calcd for $C_{12}H_{16}N_2O_{10}Na$ 371.06972 $[M + Na]^+$, found 371.06958; IR (thin film, cm^{-1}) 3374 (br, s), 2924 (m), 2887 (m), 1725 (s), 1549 (s), 1067 (br, s); UV (MeOH) λ_{max}/nm (ε/L mol^{-1} cm^{-1}) 201 (6250 ± 110), 261 (11040 ± 200). The spectral data were in agreement with the literature values.[3]

■ ASSOCIATED CONTENT

Supporting Information

Crystallographic data for compound **6** (CIF); NMR spectra for compounds **1**, **2**, and **5**−**8**; HRMS spectra of compounds **5**−**8**; HPLC-MS analyses of **1** and **8**; and ^{1}H NMR spectra of the

formation of compounds **5** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Supporting Information for Synthesis of Isoxazolin-5-one Glucosides by a Cascade Reaction

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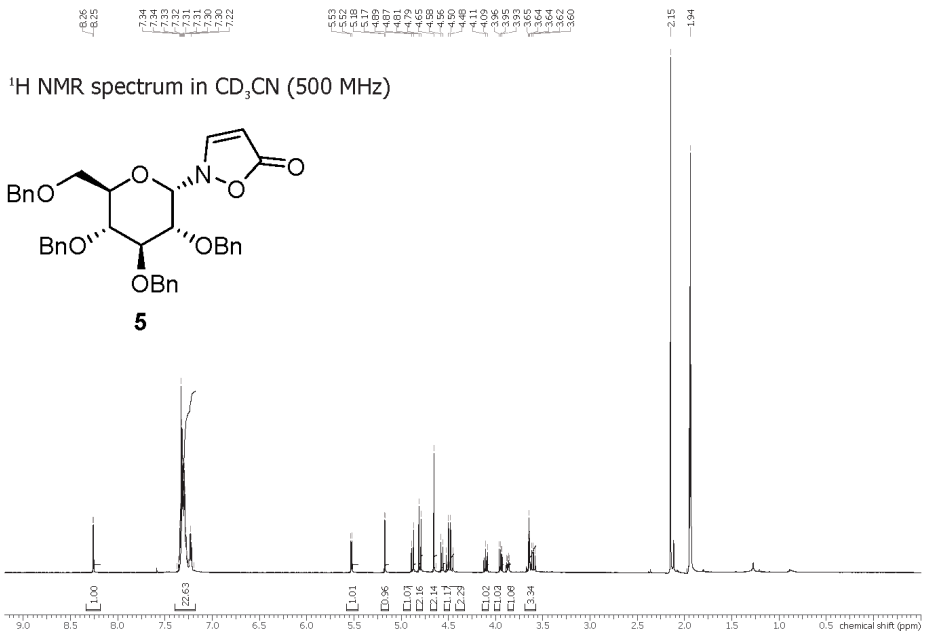
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1H NMR spectrum in CD3CN (500 MHz)
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OBn
OBn
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O
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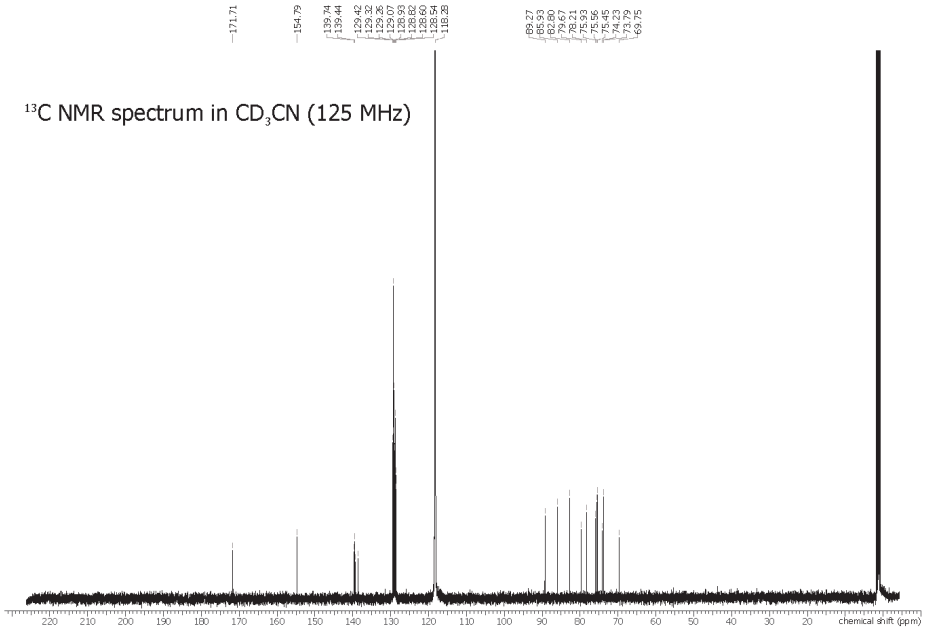
chemical shift (ppm)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85.93
82.60
79.67
78.21
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75.45
74.23
73.79
69.75
13C NMR spectrum in CD3CN (125 MHz)
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200
190
180
170
160
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70
60
50
40
30
20
chemical shift (ppm)

S2

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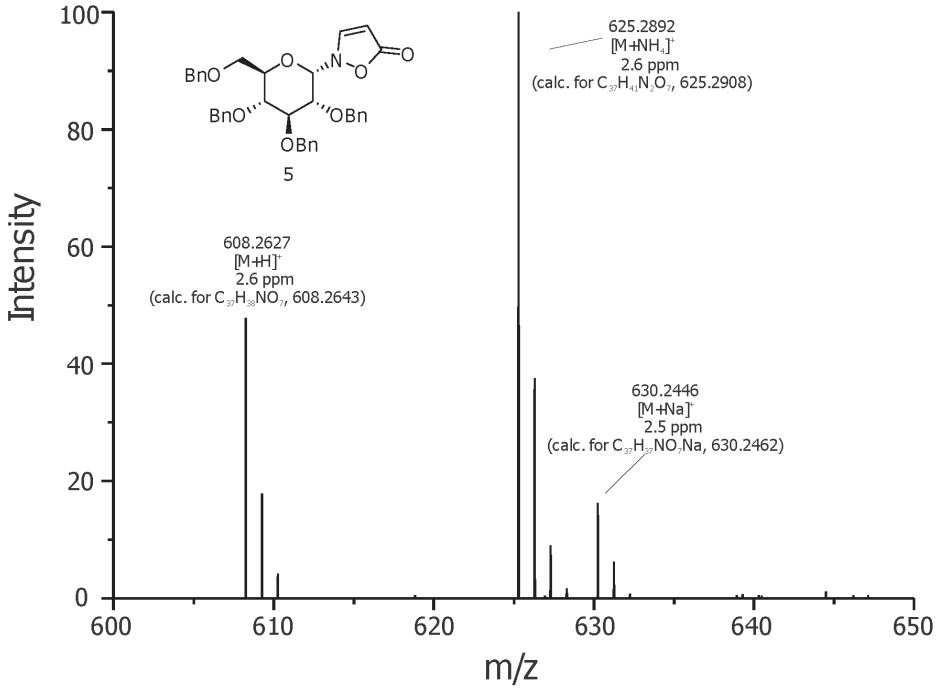

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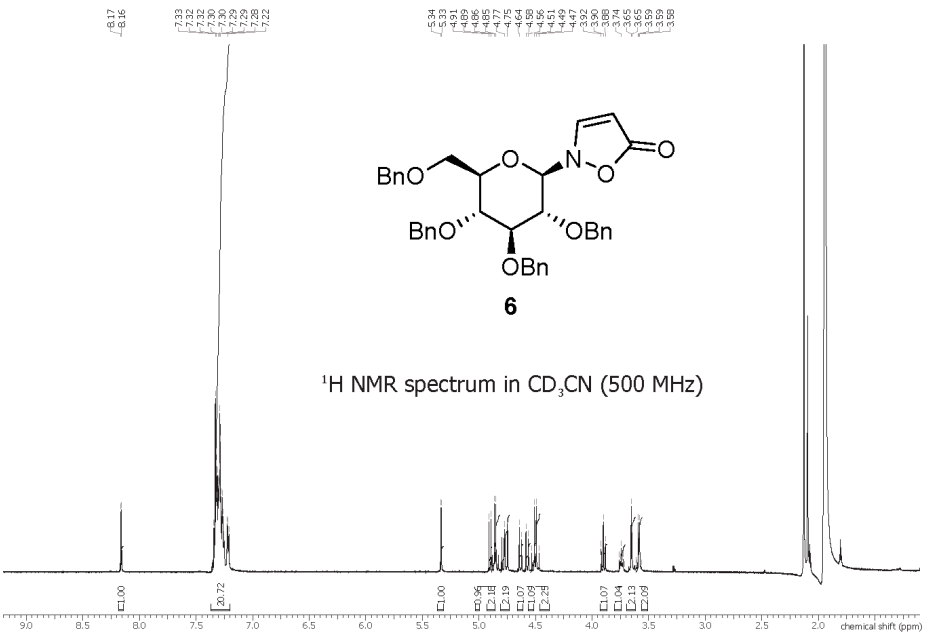
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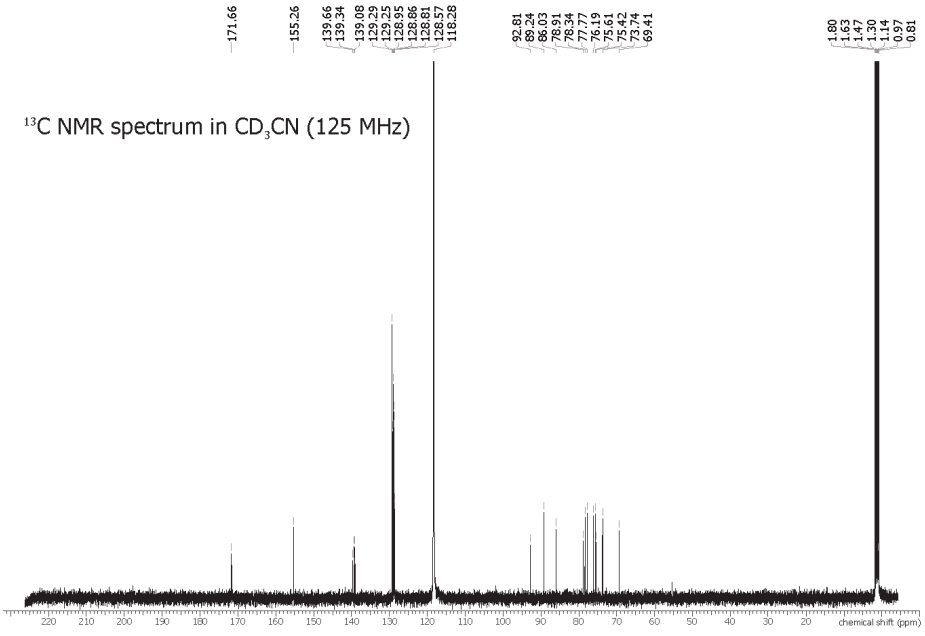
OBn
OBn
6
1H NMR spectrum in CD3CN (500 MHz)
chemical shift (ppm)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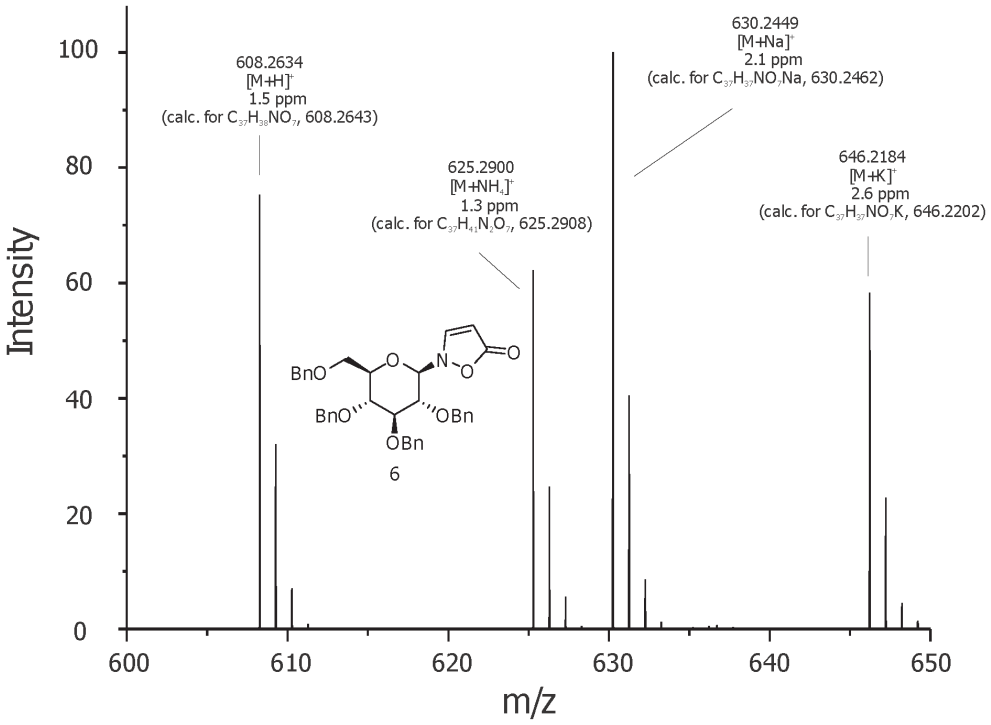
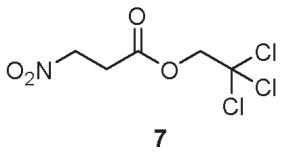


S5

^{1}H NMR spectrum in $CDCl_3$ (500 MHz)



7

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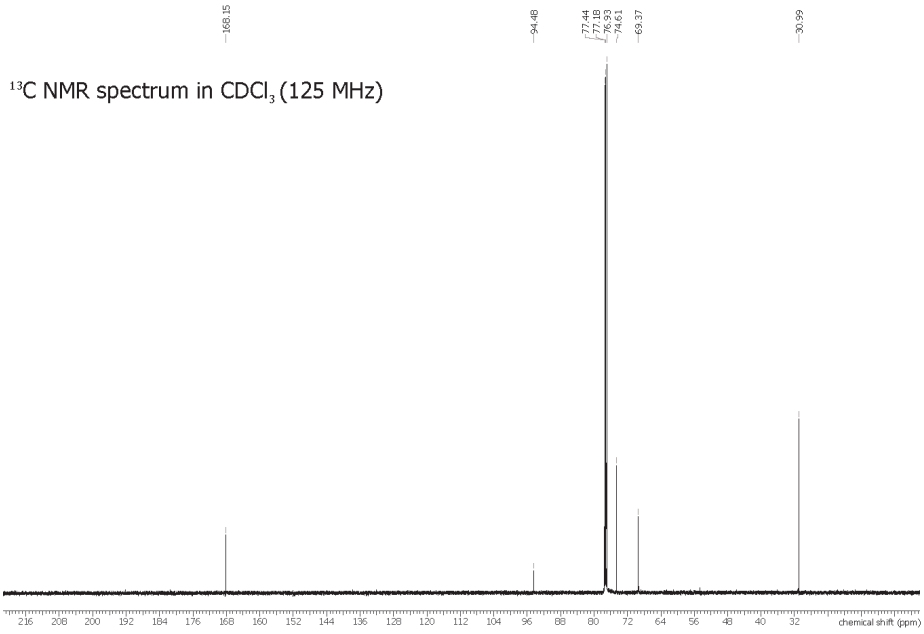
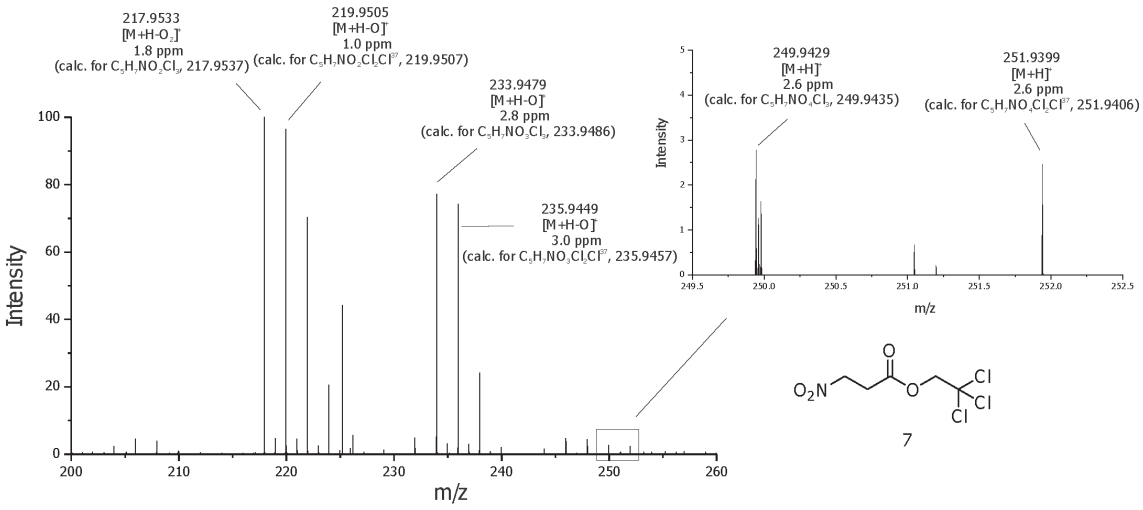


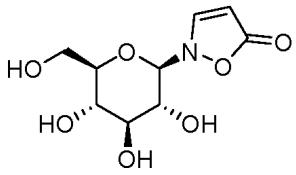
S6

MS spectrum of comp. **7**



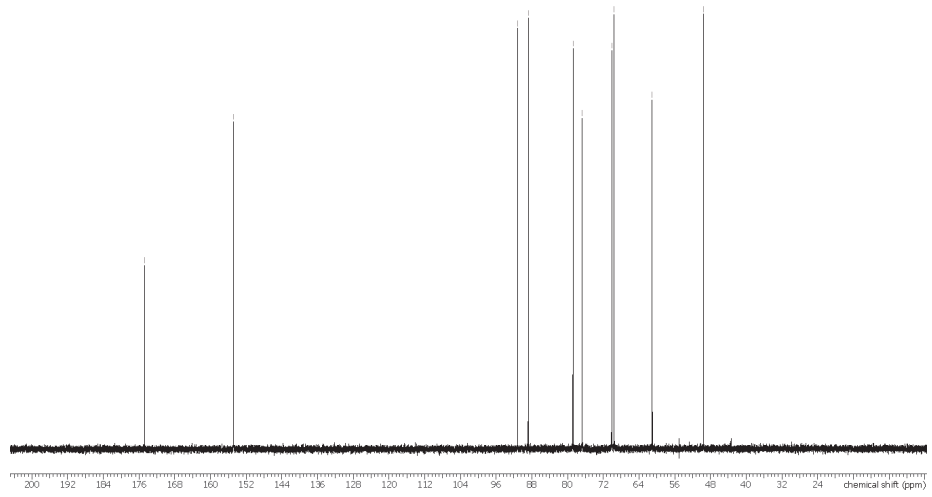
S7

^{1}H NMR spectrum in D_2O (500 MHz)

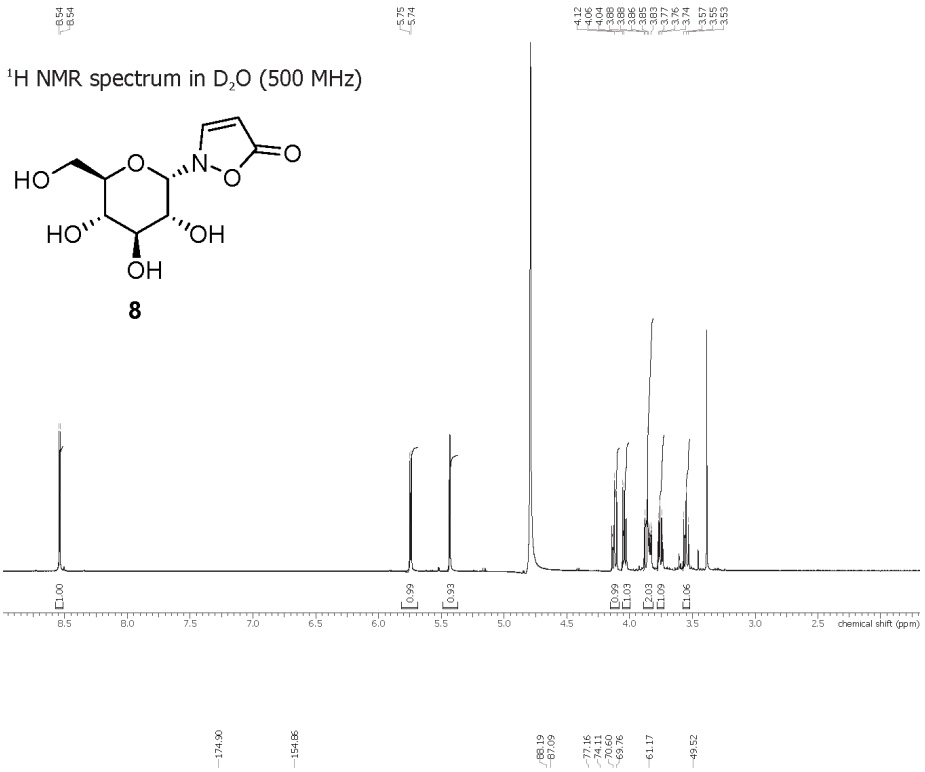


1

^{13}C NMR spectrum in D_2O (125 MHz)

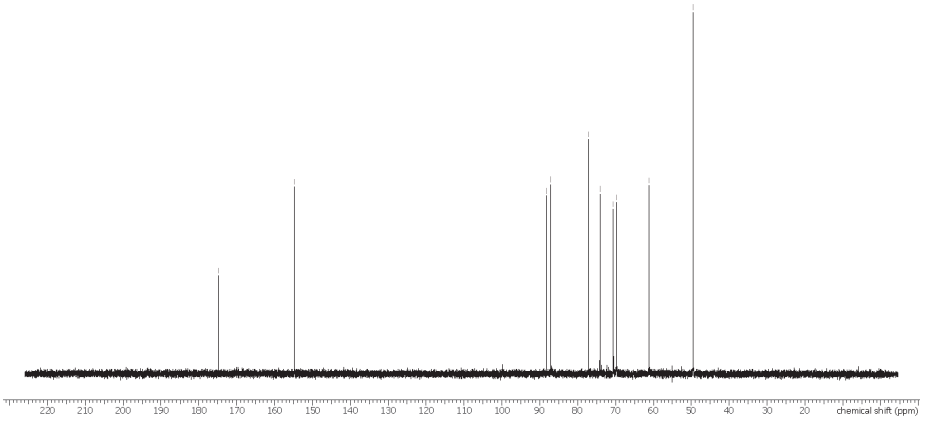


^{1}H NMR spectrum in D_2O (500 MHz)

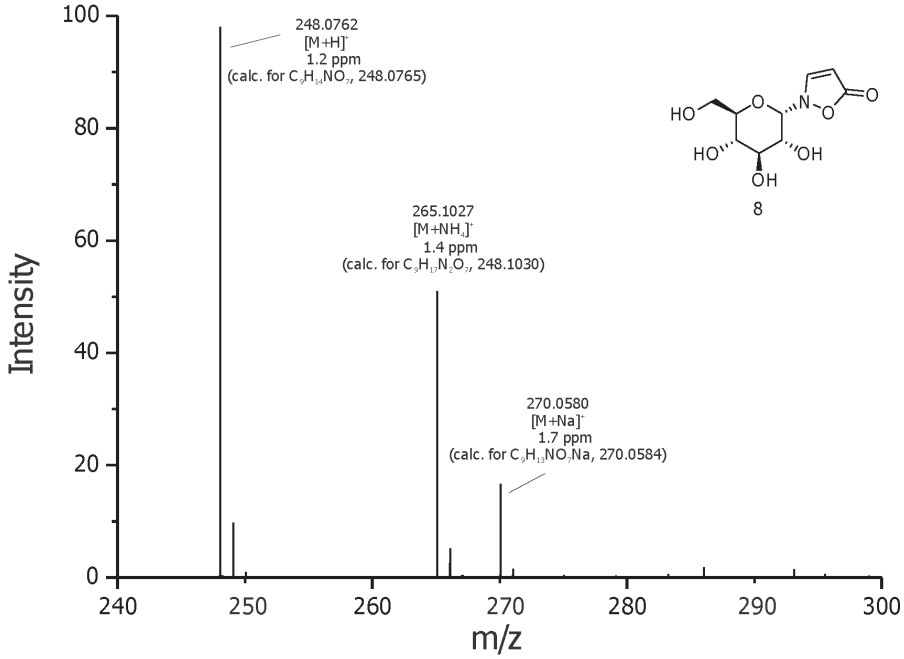


8

^{13}C NMR spectrum in D_2O (125 MHz)

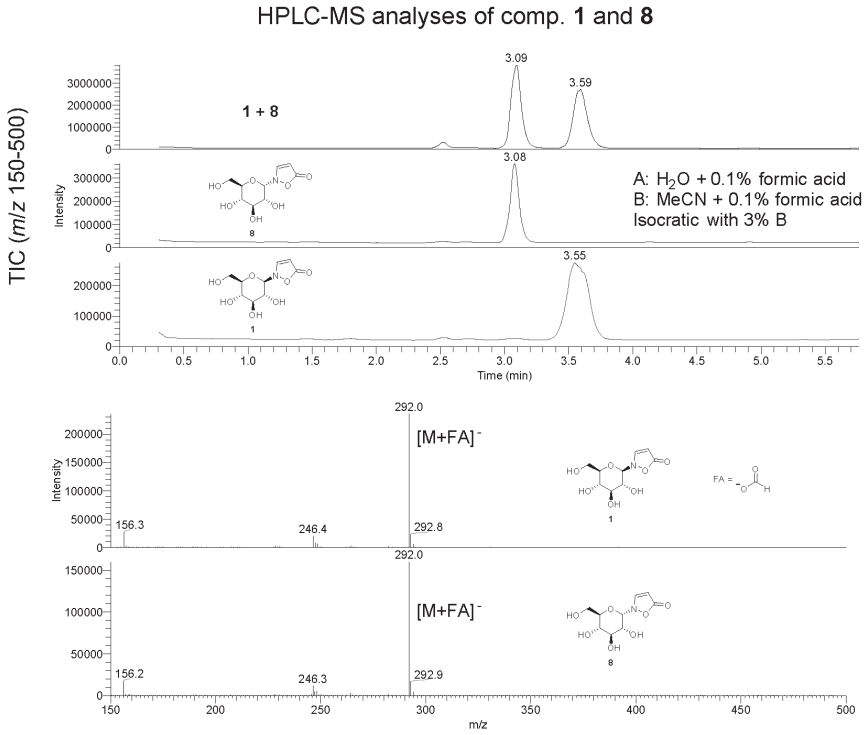


MS spectrum of comp. **8**



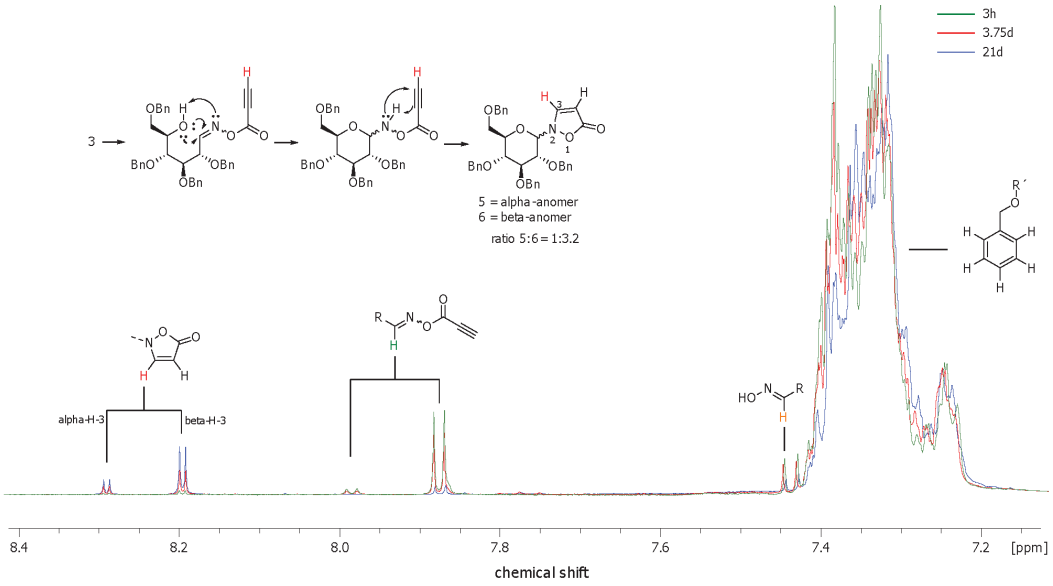
8

S10


HPLC-MS analyses of comp. 1 and 8
TIC (m/z 150-500)
1 + 8
3.09
3.59
3.08
3.55
A: H2O + 0.1% formic acid
B: MeCN + 0.1% formic acid
Isocratic with 3% B
Intensity
Time (min)
292.0
[M+FA]-
156.3
246.4
292.8
156.2
246.3
292.9
FA =
m/z

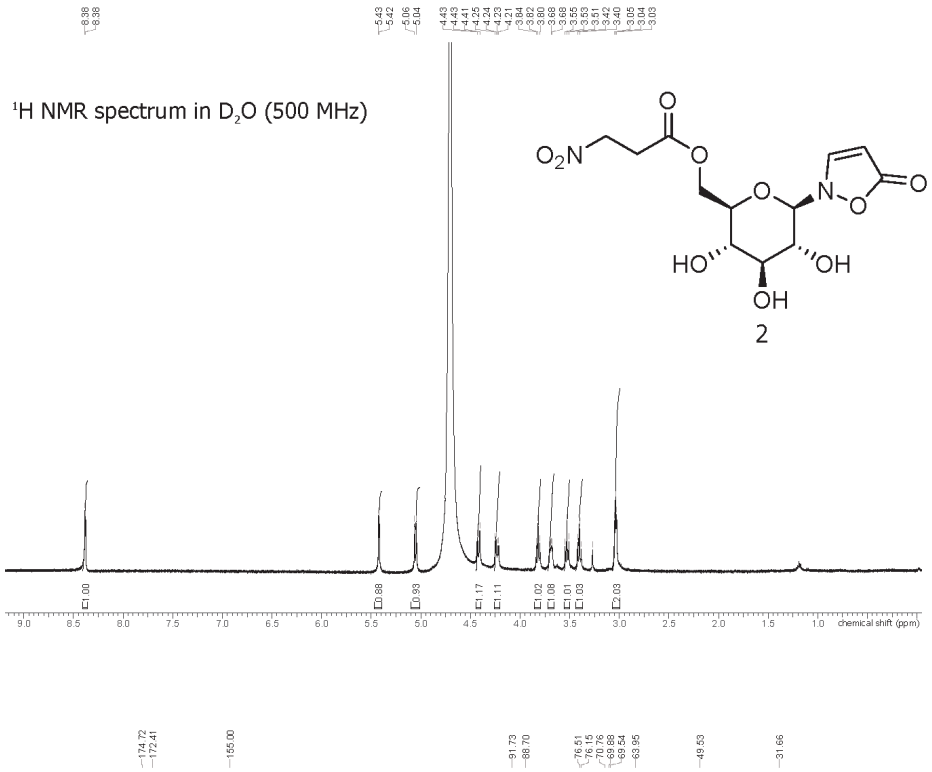
S11

^{1}H NMR spectra of the formation of compounds **5** and **6**



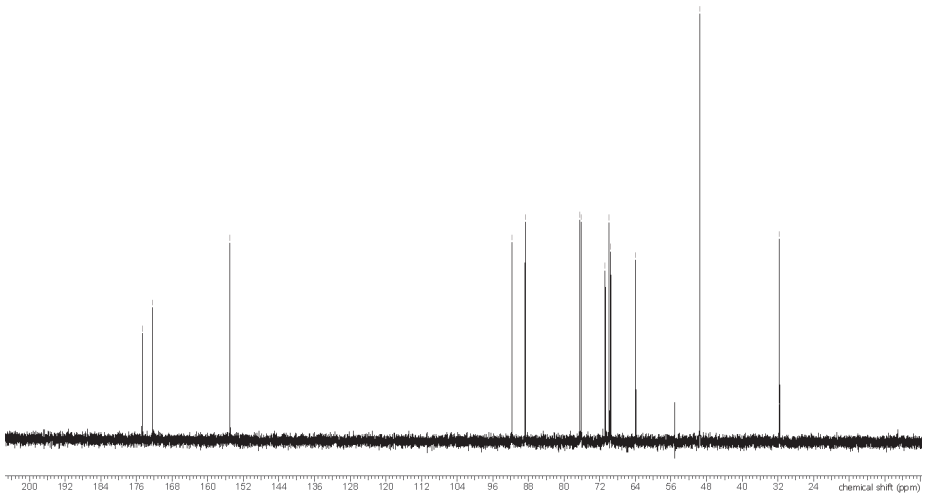
S12

^{1}H NMR spectrum in D_2O (500 MHz)



2

^{13}C NMR spectrum in D_2O (125 MHz)



S13

8.3. Manuscript 2

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Datenanalyse und -interpretation	X		X	X	X
Schreiben des Manuskripts	X				X
Publikationsäquivalente	1.0				

The subsequent manuscript has not been used in any other cumulative PhD thesis.

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Synthesis and photosensitivity of isoxazolin-5-one glycosides†

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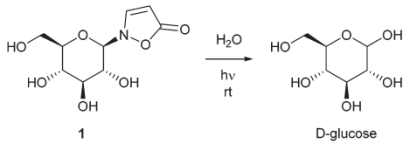
A novel procedure for the synthesis of isoxazolin-5-one glycosides starting from unprotected carbohydrates is described. The substrate scope of the one-pot synthetic protocol was explored using D-configured glucose, xylose, maltose, fructose, ribose and 2-deoxyribose. Naturally occurring 2-(β-D-glucopyranosyl)-3-isoxazolin-5-one and four novel isoxazolin-5-one glycosides derived from xylose, maltose and fructose were synthesized and purified by flash chromatography. The compounds were characterized in terms of chemical structure, photophysical properties as well as pH stability. The photohydrolysis rates of the synthesized glycosides were compared with uridine as a standard to determine the quantum yields for the photoreactions in water.

Introduction

Isoxazolin-5-one derivatives of glucose, β-aminopropionitrile and amino acids occur as secondary metabolites in different plant- and insect families.[1–14] 2-(β-D-Glucopyranosyl)-3-isoxazolin-5-one (**1**) is one of the major components of the defensive secretions of diverse leaf beetle species (Chrysomelina).[7,10–13] Seedlings of a variety of plants within the legume family (Fabaceae) contain high amounts of this compound during development and growth.[1–5] It is described that 3-unsubstituted isoxazolin-5-one derivatives show rapid hydrolysis under neutral conditions upon exposure to low intense UV light.[1,3,8] Consequently, UV irradiation corresponding to the absorption band of isoxazolin-5-one glucoside **1** results in the release of free D-glucose (Scheme 1).[1,3]

The mechanism and the efficiency of the isoxazolin-5-one decomposition in aqueous solution was studied in case of plant metabolites that derive from amino acids as well as β-aminopropionitrile.[8] Due to a lack of synthetic access, the quantum yield of the decomposition process in glucosides and the efficiency of the sugar release have not been determined so far. Furthermore, the synthesis of other isoxazolin-5-one glycosides has not been described.

Different approaches for the synthesis of glucoside **1** based on the Koenigs–Knorr substitution and a cascade reaction have previously been reported.[3,15] Both methods depend on protection and deprotection of the hydroxyl functions in the sugar moiety using acetate esters or benzyl ethers.



Scheme 1 Photoactivity of naturally occurring isoxazolin-5-one glucoside **1** in water.

In order to study the efficiency of its photoactivity and to explore its biological activity we required significant amounts of compound **1** and have developed an alternative direct synthetic route starting from D-glucose. Applying our novel synthetic approach we introduced the isoxazolin-5-one moiety into common carbohydrates to study its suitability as a general photoactive group for the release of sugars from anomeric-protected precursors.

Results and discussion

Synthesis

The novel synthetic protocol is based on a two-step one-pot strategy starting with the reaction of an unprotected carbohydrate with a free anomeric position and hydroxylamine that is freshly produced from the hydrochloride.[16] Then, the solvent is removed and water is added. Propynoic acid and DCC, both in MeCN, are added simultaneously at rt to the

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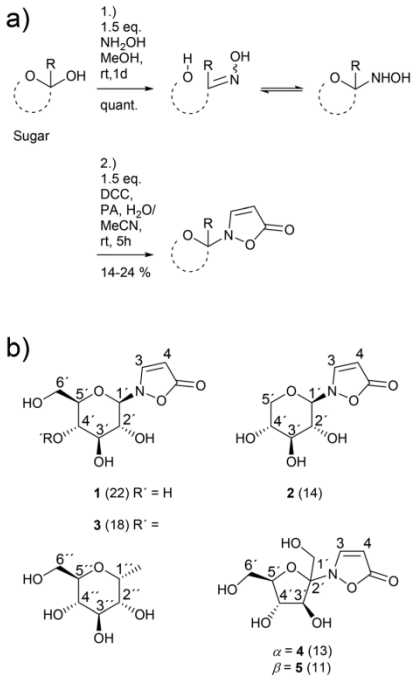
†Electronic supplementary information (ESI) available. ^{1}H and ^{13}C NMR spectra of compounds **1–5**, pH and beta-glucosidase stability plots of compound **1**, decay kinetics of uridine and compounds **1–5**. See DOI: 10.1039/c5ob00244c



Scheme 2 (a) Conditions of the synthetic protocol; sugar: D-glucose, D-xylose, D-maltose or D-fructose; quant. = quantitative; R = H, CH_2OH; DCC = dicyclohexylcarbodiimide; PA = propynoic acid; (b) structures of the isolated products **1–5**, yields are given in brackets.

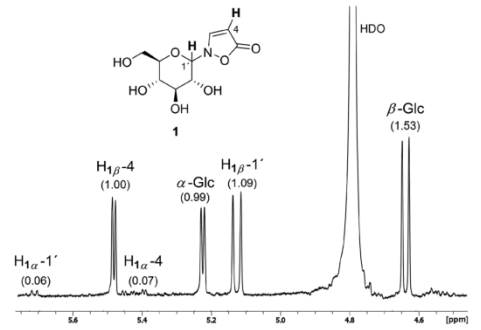
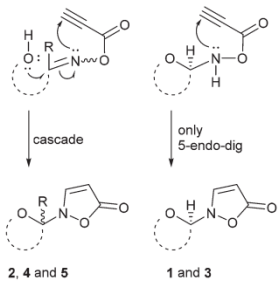


Fig. 1 Typical ^1H NMR spectrum at 400 MHz of a D_2O extract of a reaction mixture after applying the described protocol to glucose showing the anomeric protons (H-1′) as well as one of the isoxazolin-5-one protons (H-4). Integral values are given in brackets.



Scheme 3 Proposed mechanisms for the formation of the isoxazolin-5-one moiety in glycosides **1–5** after acylation of the condensation products with propynoic acid.

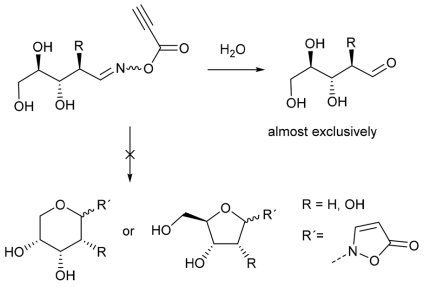
aqueous solution (Scheme 2).[17] This counterintuitive procedure results in quantitative chemoselective acylation of the *N*-hydroxy function without the need for an additional esterification catalyst. Furthermore, it allows a simple and complete separation of the sugar derivatives from the water insoluble side product 1,3-dicyclohexylurea (DCU) *via* aqueous extraction of the dried crude mixture.

The ^1H NMR spectrum of a typical reaction mixture using D-glucose (Glc) reveals the formation of the free sugar as the main product upon hydrolysis in the aqueous reaction medium (Fig. 1). The signals corresponding to the isoxazolin-5-one glucoside display the second highest intensity and show that the β-isomer is formed selectively (β/α is *ca.* 16 : 1). The total isolated yield of 2-(β-D-glucopyranosyl)-3-isoxazolin-5-one (**1**) is in the range of previous reported syntheses (here: 22%, lit.[15]: 21%). The time that is needed for the synthesis and isolation of the isoxazolin-5-one glucoside is reduced from several days to hours.[3,4,15]

The β/α-selectivity for the formation of analogous isoxazolin-5-one xylosides **2** is 4 : 1. For the corresponding maltosides **3** a ratio of 12 : 1 was determined. In case of fructose an anomeric mixture of the furanosides **4** and **5** in a ratio of *ca.* 1 : 1 was obtained. The isoxazolin-5-one ribosides and (2-deoxy)ribosides were not isolated due to product yields of less than 5% as shown by ^1H NMR measurements.

The product scope and observed β-selectivities can be understood considering the result of the condensation reaction with hydroxylamine (first step) as well as kinetic effects. In case of glucose and maltose the formation of the corresponding open chain (methanol soluble) aldoxime is the first step, followed by the crystallization of the β-*N*-pyranosylhydroxylamine in quantitative yield.[16,18] This observation explains the high selectivity of the β- over the α-anomer in case of isoxazolin-5-one glycosides derived from glucose and maltose (Scheme 3, right side).

In contrast, xylose and fructose form open chain oximes after addition of hydroxylamine in methanol almost exclusively.[16] Thus, a low stereoselectivity in the formation of isoxazolin-5-one glycosides derived from fructose and xylose is observed (Scheme 3, left side).



Scheme 4 Hydrolysis of acylated ribose- and 2-deoxyribose oxime.

In case of ribose the ^{1}H NMR spectra show that the crystalline reaction product of the condensation reaction mainly consists of the open chain oximes, too. ^{1}H NMR analysis of the reaction mixtures of ribose and 2-deoxyribose show that after acylation with propynoic acid the hydrolysis reaction is predominant (Scheme 4).

Due to the *cis*-configuration of the substituents in ribopyranosides and -furanosides we conclude that the formation of isoxazolin-5-one ribosides is kinetically disfavored against the hydrolysis reaction in the aqueous medium. This holds true for the formation of 2-deoxyribopyranosides. However, the analogous 2-deoxyribofuranosides were also not observed in adequate yields.

The purification of the isoxazolin-5-one glycosides was accomplished by low pressure flash chromatography[19] using MeCN–H_2O eluents. Due to the low solubility of the glycosides in the applied solvent mixtures, the crude mixture was extracted with water. The extract was concentrated after addition of acetonitrile onto a small amount of dry silica. The dry silica adsorbed mixture was then applied to the column and eluted.[20] The isolated products were analyzed by NMR-, HRMS-, IR-, UV- as well as optical rotation measurements.

The IR spectra of compounds **1–5** show absorptions centered at 1718–1696 cm^{-1} ($\nu_{C=O}$), 1536–1553 cm^{-1} ($\nu_{C=C}$) and 1038–1069 cm^{-1} (ν_{C-O}) being characteristic for isoxazolin-5-one glycosides.[3,15] The ^{1}H NMR spectra of compounds **1–5** show doublet signals with typical chemical shifts and coupling constants for H-3 (δ 8.45 – 8.56) and H-4 (δ 5.39 – 5.50 ppm; $^3J_{3,4}$ = 3.7 Hz). The signals of H-1′ in compounds **1–3** appear at δ 5.05–5.13 ppm showing coupling constants $^3J_{1',2'}$ in the range of 8.6–9.2 Hz. The ^{13}C NMR spectra of compounds **1–5** show chemical shifts corresponding to the isoxazolin-5-one ring in narrow ranges between δ 174.4–175.3 (C-5), 153.5–155.1 (C-3) and 89.1–91.7 (C-4) ppm. The anomeric carbon atoms (C-1′) in compounds **1–3** show absorption at δ 88.6–89.4 ppm. These data are in excellent agreement with literature values.[3,4,9,15] ^{1}H NMR spectra of the fructosides **4** and **5** provide chemical shifts of 4.61 and 4.36 ppm that correspond to the H-3′position. The coupling constants $^3J_{3',4'}$ are 5.0 (α-anomer) and 8.4 Hz (β-anomer) respectively. The chemical shifts of H_B-6′ equal 3.73 ($^3J_{5',6'}$ = 4.6 Hz, comp. **4**) and 3.75 ppm ($^3J_{5',6'}$ = 6 Hz, comp. **5**). The ^{13}C NMR spectra of compounds **4** and **5** show signals at δ 100.3 (C-2′, α-anomer **4**) and 97.5 ppm (C-2′, β-anomer **5**). The signals at 61.1 (comp. **4**) and δ 61.5 ppm (comp. **5**) correspond to the C-6′-position. Moreover, the optical rotations are 68.4 (α-anomer **4**) and −27.8° (β-anomer **5**). All of these data are in excellent agreement with previous reported results for *N*-fructofuranosides.[21–23]

Having access to isoxazolin-5-one glycosides we studied their pH dependent stability[1,3,4] as well as the stability of compound **1** against β-glucosidase. ^{1}H NMR and UV measurements of solutions of compound **1** in D_2O showed that the *N*-glycosidic moiety is inert in a range of 7 ≥ pH ≥ 0 at rt, which is untypical for many kinds of glycosidic bonds. The π-conjugation in the aromatic isoxazolin-5-one ring reduces the basicity of the free electron pair of the nitrogen atom and thus the stability against electrophilic attack is increased. Consequently, β-glucosidase from almonds could not cleave the *N*-glycosidic bond in compound **1** at pH = 5 and rt due to its catalytic mechanism.[24] In contrast, the absorption band disappears rapidly at pH > 7. The decay rates increase with ascending pH (see ESI, Fig. S2†). ^{1}H NMR spectra in D_2O show that the *N*-glycosidic bond is inert at pH > 7 while the double bond signals H-3 and H-4 disappear due to a β-addition of an hydroxyl anion to the α,β-unsaturated carbonyl structure. After re-acidification the *N*-glycosidic bond is cleaved and the free sugar is formed as expected due to the increased basicity of the lone electron pair of the nitrogen atom.

Photophysical properties

All of the novel glycosides show molar extinction coefficients around 10 800 M^{-1} cm^{-1} at wavelengths of maximum extinction between 260 and 266 nm (Table 1). Upon irradiation with a low intensity UV lamp (λ_{max} = 254 nm) ^{1}H NMR studies in D_2O show a quantitative decay of the glycosides releasing the sugar from which they were derived. The quantum yield of this photohydrolysis reaction was determined by comparison of the decomposition kinetics of the isolated compounds **1–5** with uridine (U) as a standard. Equally concentrated solutions of the glycosides were placed in a cuvette and irradiated with a weak UV lamp. Simultaneously the absorbances of the solutions have been measured over the time. The intensity of the light source was determined (Fig. 2).

Table 1 S = substance, U = uridine; wavelengths of maximum absorption (λ_{max}), molar extinction coefficients (ε), and quantum yields (Φ) of the photohydrolysis reactions

S	λ_{max}/nm	$\varepsilon/10^3$ M^{-1} cm^{-1}	Φ
U	260	9.7	0.019 ± 0.001[25]
1	260	10.8	0.324 ± 0.018
2	260	10.8	0.327 ± 0.019
3	261	10.8	0.299 ± 0.017
4	266	10.8	0.284 ± 0.016
5	265	10.8	0.275 ± 0.016

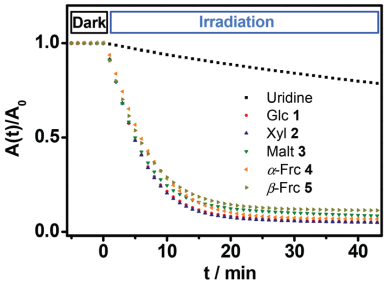


Fig. 2 Decay kinetics for the photohydrolysis of the glycosides; black: uridine, red: β-glucoside **1**, blue: β-xyloside **2**, green: β-maltoside **3**, orange: α-fructofuranoside **4**, yellow: β-fructofuranoside **5**; λ = 261 nm; $c_0 = 3.5 \times 10^{-5}$ M; $I_{261\ nm} = 0.18$ mW cm^{-2}; $d_{lamp} = 5$ cm; stirred; over the first five minutes absorbances were measured without illumination (dark).

The first five minutes without illumination show a constant absorption with no significant change in case of all glycosides. After starting the irradiation with the low intense UV lamp all of the curves show a decrease in absorbance that is significantly faster compared to the decay of uridine (U) under the same conditions. The slopes of the curves at the beginning of the irradiation were determined. *Via* comparison with the standard the quantum yields of the photoreactions were calculated (see Experimental section). The determined quantum yields lie in a range of 0.275 to 0.327 (Table 1).

These values are in the same order of magnitude as those reported for quantum yields of naturally occurring amino acid derivatives of isoxazolin-5-one (0.5).[8] Thus, the described compounds **1–5** show a rapid and very efficient release of their corresponding free sugars at room temperature upon irradiation with very weak UV light.

Conclusion

A novel synthetic protocol has been developed for the synthesis of isoxazolin-5-one glycosides that does not depend on the use of protecting groups. The reaction was successfully applied to glucose, xylose, maltose and fructose to afford the corresponding glycosides **1–5**. Although this one-pot strategy gives only low yields, it can be used to provide rapidly a significant amount of the described glycosides. The product scope and selectivity of the reaction have been discussed. The main products were isolated and characterized in terms of their chemical structure, stability and photophysical properties. High quantum yields of the photohydrolysis reactions have been observed. As a consequence of the described photolability as well as the stability against low pH and enzymatic hydrolysis, we conclude that the isoxazolin-5-one moiety provides potential for the use as a photoactive and bioorthogonal protecting group for the anomeric position in glycosides.

Experimental section

General

Melting points were determined with a capillary melting point apparatus. Infrared spectra were measured with an IR spectrometer in a range of 700–4000 cm^{-1} in transmission mode with a spectral resolution of 6 cm^{-1}. Optical rotations were measured at 589 nm in water (temperatures are given). NMR spectra were measured using a spectrometer operating at 400 MHz (^{1}H) and 100 MHz (^{13}C). Chemical shifts (δ) are quoted in parts per million (ppm) and are referenced to the signal of residual protonated solvent (HDO at δ 4.79 ppm). Acetonitrile was added as a reference for ^{13}C NMR spectra in D_2O. Assignment of peaks was carried out using 2D NMR experiments (COSY, HSQC, and HMBC). The multiplicities are given as follows: d, doublet; dd, doublet of doublets; m, multiplet. High-resolution mass spectra were recorded on a UHR-qTOF mass spectrometer.

The columns for preparative chromatography were packed by pouring a suspension of silica gel (0.03–0.063 mm) in the eluent into the column containing 40 ml of the eluent. When the sedimentation process was completed the column was opened and additional pressure was slowly increased up to 1.2 bar. The column was washed with 300 ml of solvent before adding the silica-adsorbed mixture. The separations were carried out at 1.1 bar additional pressure.

Thin-layer chromatography was performed on TLC silica gel 60 F254 aluminum sheets. Compounds were visualized using an UV lamp with a maximum emission at 254 nm. All reagents and solvents were purchased in the highest purity that was commercially available and used without further purification.

Photophysical measurements

Absorptions were recorded on a UV spectrophotometer with a spectral resolution of 2 nm at 261 nm and room temperature. Quantum yields and photon flux were determined by using commercially available uridine (99%) as a chemical actinometer according to eqn (1).[8,25] The accuracies were estimated using eqn (2). The intensity of the UV lamp was determined applying eqn (3).

$$\Phi = \left(\frac{A}{t}\right)\frac{V}{q\varepsilon d} \quad (1)$$

$$\Delta\Phi = \frac{V}{q\varepsilon d}\sqrt{\left(\Delta\left(\frac{A}{t}\right)\right)^2 + \left(-\left(\frac{A}{t}\right)\frac{\Delta q}{q}\right)^2 + \left(-\left(\frac{A}{t}\right)\frac{\Delta\varepsilon}{\varepsilon}\right)^2} \quad (2)$$

$$I = \frac{qN_A hc}{F\lambda} \quad (3)$$

Φ = Quantum yield, $\left(\frac{A}{t}\right)$ = slope of the change in absorption over short time periods in s^{-1}, V = volume of the irradiated solution in l, q = photon flux in Einstein s^{-1}, ε = molar extinction coefficient in l mol^{-1} cm^{-1}, d = length of the cuvette in cm, N_a = Avogadro constant in mol^{-1}, h = Planck constant in J s, c = speed of light in ms^{-1}, F = area of irradiation (1 cm^2),

λ = wavelength (261 nm), I = intensity in W cm^{-2} and ΔX = the standard deviation of X. The substrates were dissolved in a buffer (Na_2HPO_4–NaH_2PO_4; 50 mM) at pH 7 in a concentration of (3.5 ± 0.2) × 10^{-5} M. The total volume of the solution was (1.63 ± 0.03) ml. A UV lamp (λ_{max} = 254 nm, 6 W) was used for the illumination. The cuvette (d = 1 cm) was irradiated vertically to the measurement geometry at a distance (d_{lamp}) of 5 cm. The solution was stirred *via* magnetic induction. The absorption at a wavelength of 261 nm was measured time dependently and simultaneously to irradiation and stirring. The observed molar extinction coefficients are in excellent agreement with the literature results.[1–4,6,8,15,26]

General synthetic procedure

To a stirred solution of 209 mg (3 mmol, 1.5 eq.) hydroxylamine hydrochloride in 2 ml dry methanol 314 mg (2.8 mmol, 1.4 eq.) potassium *tert*-butoxide were added in portions at 0 °C under stirring. After 1 h at rt the solution was filtered under vacuum, washed with 1 ml of dry methanol and 2 mmol sugar were added. After 1 d of stirring at rt the solvent was removed under reduced pressure at 40 °C. The dry residue was dissolved in 1 ml of water. Under stirring 0.5 ml of a freshly prepared solution of DCC in MeCN (c = 0.4 M, 0.1 eq.) was added at once. Then further 7 ml of DCC in MeCN (c = 0.4 M, 1.4 eq.) and 7 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 × 5 ml). To the filtrate 150 ml of MeCN were added and the solvents were removed at 25 °C and 75 mbar. Then 1.25 g of dry silica and 150 ml MeCN were added. The solvents were removed again at 25 °C and 75 mbar to yield a dry crude mixture.

Column chromatography and analytical data of 2-(β-D-glucopyranosyl)-3-isoxazolin-5-one (**1**): The dry mixture was applied to a column and eluted (MeCN–H_2O 55 : 1, silica). The product fractions were combined and concentrated to yield 109 mg (0.44 mmol, 22%) of **1** as a colorless powder.

$[\alpha]_D^{24}$ +14.6 (c 0.81, H_2O); R_f(MeCN–H_2O 55 : 1) = 0.15; ^{1}H NMR (400 MHz, D_2O) δ 8.47 (d, $^3J_{3,4}$ = 3.7 Hz, 1H, H-3), 5.47 (d, $^3J_{3,4}$ = 3.7 Hz, 1H, H-4), 5.12 (d, $^3J_{1',2'}$ = 9.2 Hz, 1H, H-1′), 3.90–3.85 (m, 2H, HA-6′ and H-2′), 3.71 (dd, $^2J_{6'A,6'B}$ = 12.5 Hz, $^3J_{5',6'B}$ = 6.9 Hz, 1H, HB-6′), 3.62– 3.54 (m, 2H, H-3′ and H-5′), 3.46 (dd, $^3J_{3',4'}$ = $^3J_{4',5'}$ = 9.5 Hz, 1H, H-4′); ^{13}C NMR (100 MHz, D_2O) δ 174.8 (C-5), 154.9 (C-3), 91.2 (C-4), 88.9 (C-1′), 78.8 (C-5′), 76.8 (C-3′), 70.0 (C-2′), 69.6 (C-4′), 61.0 (C-6′); HRMS (ESI-TOF) m/z calcd for $C_9H_{14}NO_7$ 248.0765 [M + H]$^+$, found 248.0758 ($\Delta m/z$ 2.8 ppm); IR (thin film, cm^{-1}) 3382 (br, s), 2922 (m), 1718 (s, br), 1546 (s), 1069 (br, s); UV (H_2O) λ_{max}/nm (ε/L mol^{-1} cm^{-1}) 260 (10 800 ± 200); mp 147–149 °C (decomp.).

Column chromatography and analytical data of 2-(β-D-xylopyranosyl)-3-isoxazolin-5-one (**2**): MeCN–H_2O 85 : 1; yield: 58 mg (0.28 mmol, 14%); colorless oil.

$[\alpha]_D^{24}$ −53.2 (c 0.84, H_2O); R_f(MeCN–H_2O 85 : 1) = 0.22; ^{1}H NMR (400 MHz, D_2O) δ 8.45 (d, $^3J_{3,4}$ = 3.7 Hz, 1H, H-3), 5.49 (d, $^3J_{3,4}$ = 3.7 Hz, 1H, H-4), 5.05 (d, $^3J_{1',2'}$ = 9.2 Hz, 1H, H-1′), 3.99 (dd, $^2J_{5'A,5'B}$ = 11.6 Hz, $^3J_{4',5'A}$ = 5.5 Hz, 1H, H_A-5′), 3.87 (dd, $^3J_{2',3'}$ = $^3J_{1',2'}$ = 9.2 Hz, 1H, H-2′), 3.67 (m, 1H, H-4′), 3.55 (dd, $^3J_{2',3'}$ = $^3J_{3',4'}$ = 9.3 Hz, 1H, H-3′), 3.42 (dd, $^2J_{5'A,5'B}$ = $^3J_{4',5'B}$ = 11.0 Hz, 1H, H_B-5′); ^{13}C NMR (100 MHz, D_2O) δ 174.7 (C-5), 155.1 (C-3), 91.7 (C-4), 89.4 (C-1′), 76.8 (C-3′), 69.9 (C-2′), 69.2 (C-4′), 67.9 (C-5′); HRMS (ESI-TOF) m/z calcd for $C_8H_{12}NO_6$ 218.0659 [M + H]$^+$, found 218.0658 ($\Delta m/z$ 0.5 ppm); IR (thin film, cm^{-1}) 3374 (br, s), 1717 (s, br), 1547 (s), 1055 (s); UV (H_2O) λ_{max}/nm (ε/L mol^{-1} cm^{-1}) 260 (10 800 ± 200).

Column chromatography and analytical data of 2-(β-D-maltopyranosyl)-3-isoxazolin-5-one (**3**): MeCN–H_2O 7 : 1; yield: 148 mg (0.36 mmol, 18%); colorless powder.

$[\alpha]_D^{25}$ +105.4 (c 0.61, H_2O); R_f(MeCN–H_2O 7 : 1) = 0.26; ^{1}H NMR (400 MHz, D_2O) δ 8.46 (d, $^3J_{3,4}$ = 3.7 Hz, 1H, H-3), 5.48 (d, $^3J_{3,4}$ = 3.7 Hz, 1H, H-4), 5.43 (d, $^3J_{1'',2''}$ = 3.9 Hz, 1H, H-1″), 5.13 (d, $^3J_{1',2'}$ = 8.6 Hz, 1H, H-1′), 3.94–3.82 (m, 4H), 3.78 − 3.65 (m, 6H), 3.58 (dd, $^3J_{2'',3''}$ = 9.9 Hz, $^3J_{1'',2''}$ = 3.9 Hz, 1H, H-2″), 3.41 (dd, $^3J_{5',6A'}$ = $^2J_{6A',6B'}$ = 9.4 Hz, 1H, H_A-6′); ^{13}C NMR (100 MHz, D_2O) δ 174.8 (C-5), 154.8 (C-3), 100.2 (C-1″), 91.3 (C-4), 88.6 (C-1′), 77.4, 77.2, 76.4, 73.4, 73.4, 72.3 (C-2″), 69.9, 69.8, 61.1, 61.0; HRMS (ESI-TOF) m/z calcd for $C_{15}H_{24}NO_{12}$ 410.1293 [M + H]$^+$, found 410.1280 ($\Delta m/z$ 3.2 ppm); IR (thin film, cm^{-1}) 3367 (br, s), 1717 (s, br), 1594 (s), 1553 (s), 1038 (br, s); UV (H_2O) λ_{max}/nm (ε/L mol^{-1} cm^{-1}) 261 (10 760 ± 200); mp 195–200 °C (decomp.).

Column chromatography and analytical data of 2-(α-D-fructofuranosyl)-3-isoxazolin-5-one (**4**): DCM–MeOH 5 : 1 and MeCN–H_2O 35 : 1; yield: 65 mg (0.26 mmol, 13%); colorless oil.

$[\alpha]_D^{25}$ +68.4 (c 0.66, H_2O); R_f(MeOH–DCM 1 : 5) = 0.30; ^{1}H NMR (400 MHz, D_2O) δ 8.56 (d, $^3J_{3,4}$ = 3.7 Hz, 1H, H-3), 5.43 (d, $^3J_{3,4}$ = 3.5 Hz, 1H, H-4), 4.61 (d, $^3J_{3',4'}$ = 5.0 Hz, 1H, H-3′), 4.15 – 4.09 (m, 2H, H-4′ and H-5′), 3.93–3.83 (m, 3H, H-1′ and H_A-6′), 3.73 (dd, $^2J_{6'A,6'B}$ = 12.7 Hz, $^3J_{5',6'B}$ = 4.6 Hz, 1H, H_B-6′); ^{13}C NMR (100 MHz, D_2O) δ 175.3 (C-5), 153.7 (C-3), 100.3 (C-2′), 89.6 (C-4), 83.8 (C-4′ or C-5′), 79.9 (C-3′), 76.3 (C-4′ or C-5′), 61.5 (C-1′), 61.1 (C-6′); HRMS (ESI-TOF) m/z calcd for $C_9H_{14}NO_7$ 248.0765 [M + H]$^+$, found 248.0755 ($\Delta m/z$ 4.0 ppm); IR (thin film, cm^{-1}) 3366 (br, s), 1717 (s, br), 1541 (s), 1058 (br, s); UV (H_2O) λ_{max}/nm (ε/L mol^{-1} cm^{-1}) 266 (10 820 ± 200).

Column chromatography and analytical data of 2-(β-D-fructofuranosyl)-3-isoxazolin-5-one (**5**): DCM–MeOH 5 : 1 and MeCN–H_2O 35 : 1; yield: 55 mg (0.22 mmol, 11%); colorless oil.

$[\alpha]_D^{25}$ −27.8 (c 0.85, H_2O); R_f(MeOH–DCM 1 : 5) = 0.20; ^{1}H NMR (400 MHz, D_2O) δ 8.56 (d, $^3J_{3,4}$ = 3.7 Hz, 1H, H-3), 5.39 (d, $^3J_{3,4}$ = 3.7 Hz, 1H, H-4), 4.36 (d, $^3J_{3',4'}$ = 8.4 Hz, 1H, H-3′), 4.26 (dd, $^3J_{3',4'}$ = $^3J_{4',5'}$ = 8.4 Hz, 1H, H-4′), 4.06 (d, $^2J_{1A',1B'}$ = 12.7 Hz, 1H, H_A-1′), 3.97 (d, $^2J_{1A',1B'}$ = 12.7 Hz, 1H, H_B-1′), 3.92–3.86 (m, 2H, H-5′ and H_A-6′), 3.75 (dd, $^2J_{6'A,6'B}$ = 12.9 Hz, $^3J_{5',6'B}$ = 6.0 Hz, 1H, H_B-6′); ^{13}C NMR (100 MHz, D_2O) δ 175.0 (C-5), 153.5 (C-3), 97.5 (C-2′), 89.1 (C-4), 82.1 (C-5′), 78.4 (C-3′), 73.8 (C-4′), 62.6 (C-1′), 61.5 (C-6′); HRMS (ESI-TOF) m/z calcd for $C_9H_{14}NO_7$ 248.0765 [M + H]$^+$, found 248.0756 ($\Delta m/z$ 3.6 ppm); IR (thin film, cm^{-1}) 3360 (br, s), 1696 (s, br), 1536 (s), 1046 (s); UV (H_2O) λ_{max}/nm (ε/L mol^{-1} cm^{-1}) 265 (10 780 ± 200).

Author information

TB and WB planned the experiments. TB and PK synthesized the compounds. TB performed the photophysical characterizations. TB, SHvR and CP performed NMR analyses. TB, SHvR and WB wrote the manuscript.

Conflict of interest

The authors declare no competing financial interest.

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Notes and references

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Supporting Information for Synthesis and Photosensitivity of Isoxazolin-5-one Glycosides

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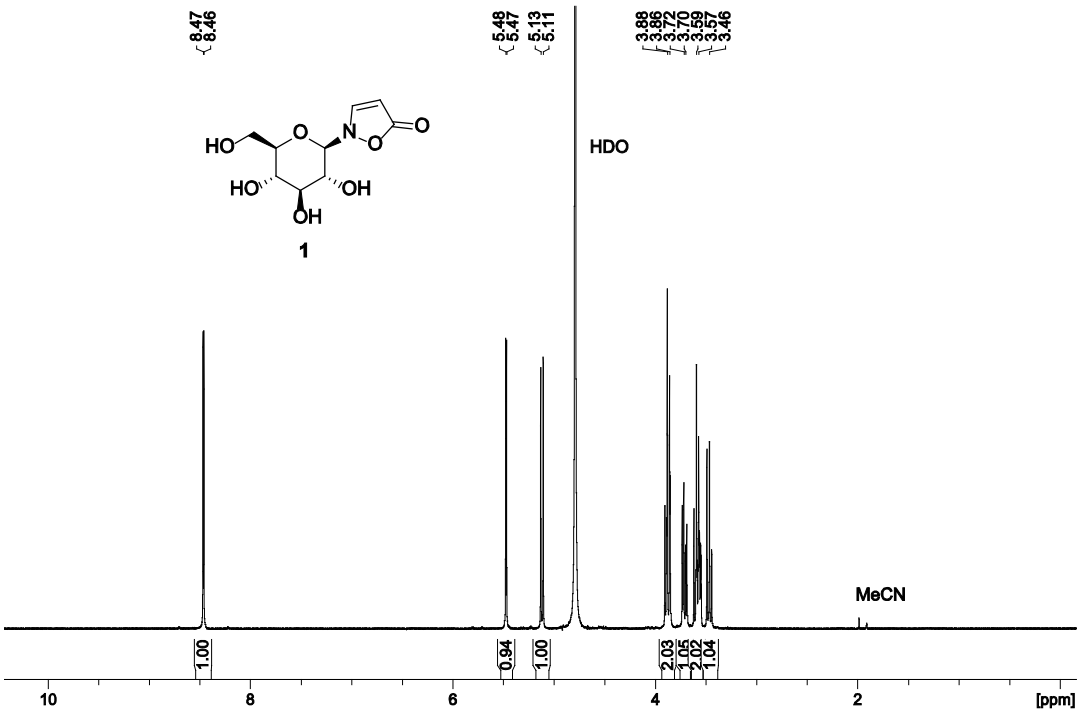
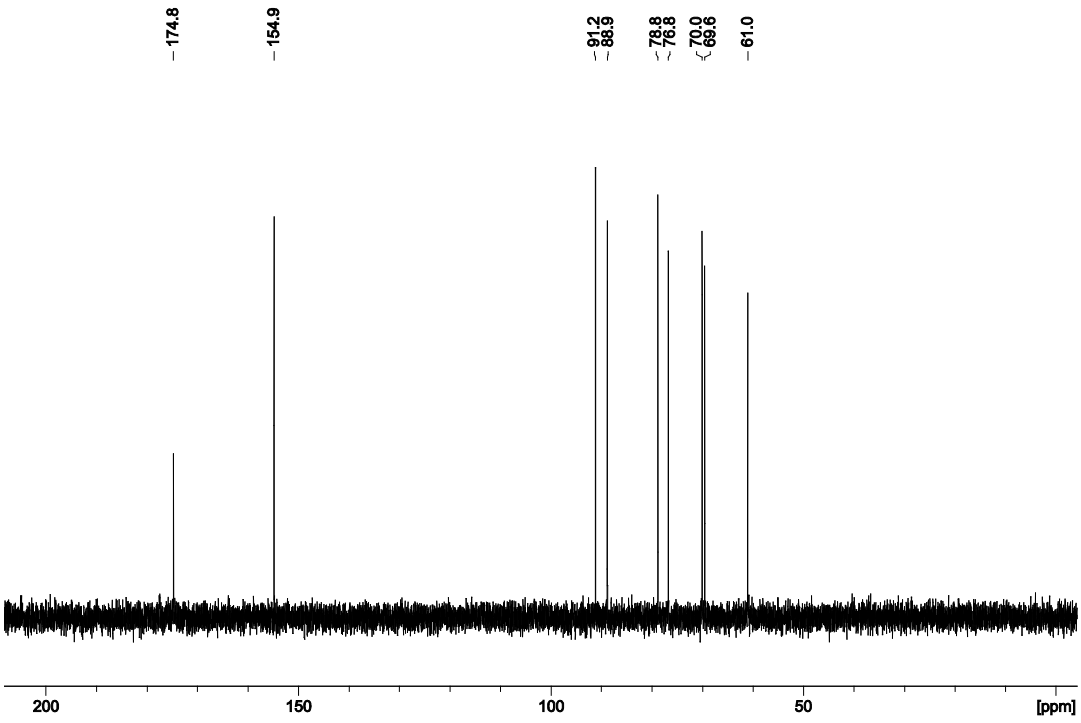
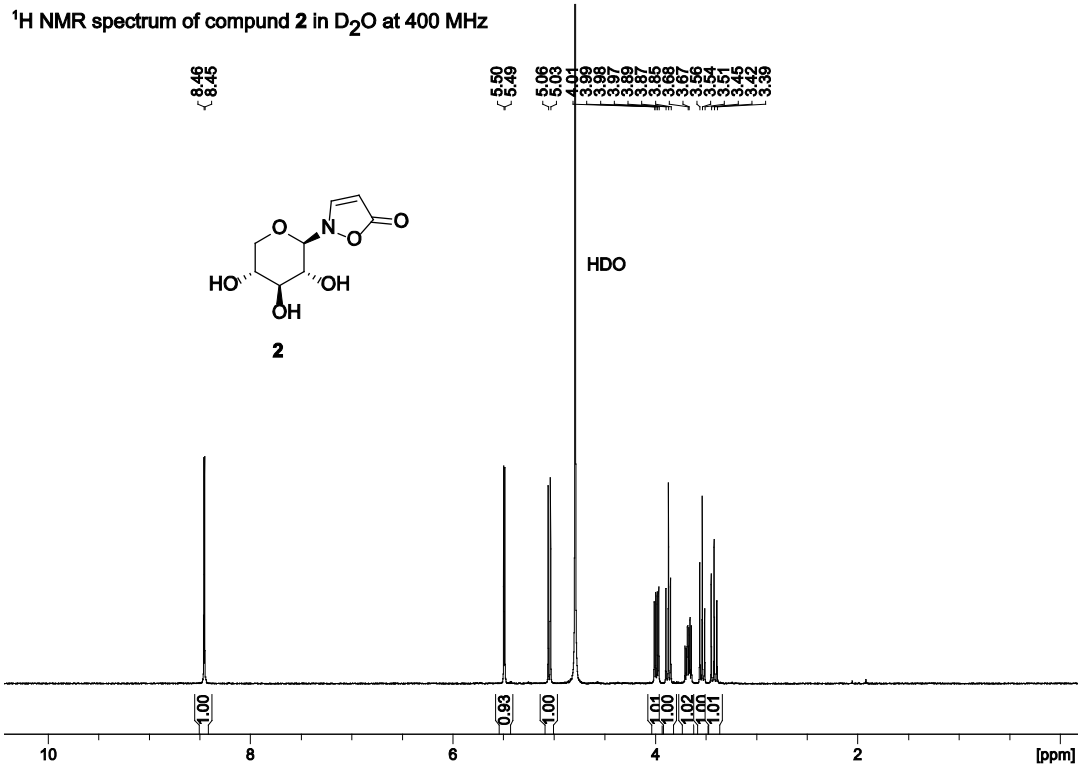
^{1}H NMR spectrum of compound **1** in D_2O at 400 MHz



^{13}C NMR spectrum of compound **1** in D_2O at 100 MHz

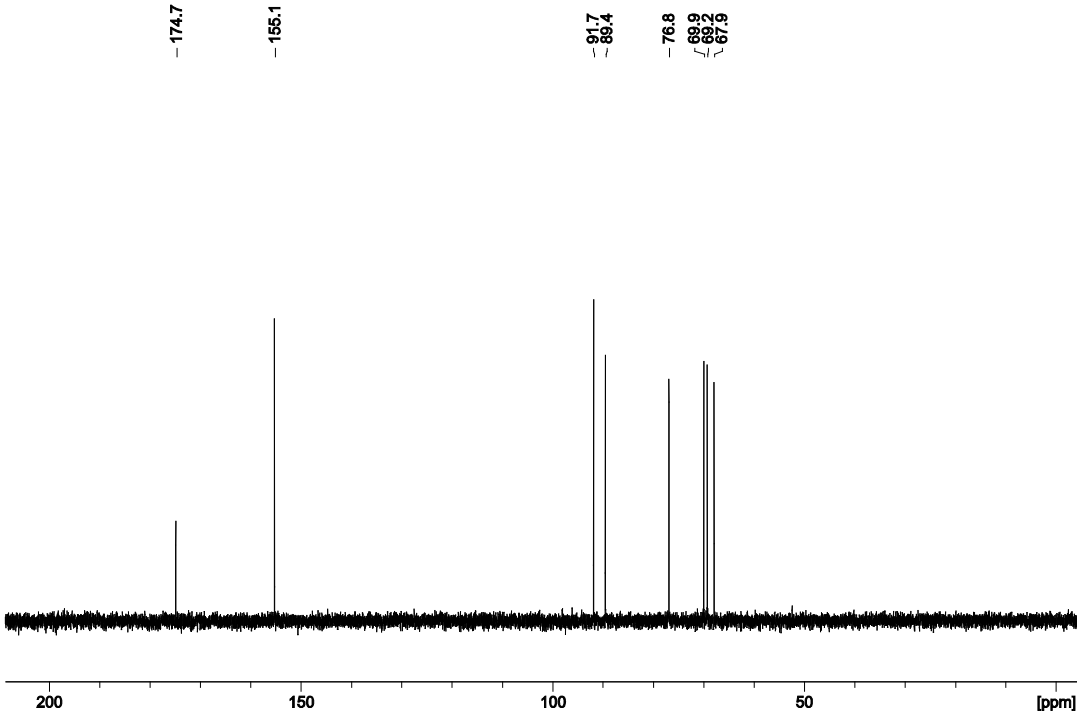


^{1}H NMR spectrum of compund **2** in D_2O at 400 MHz

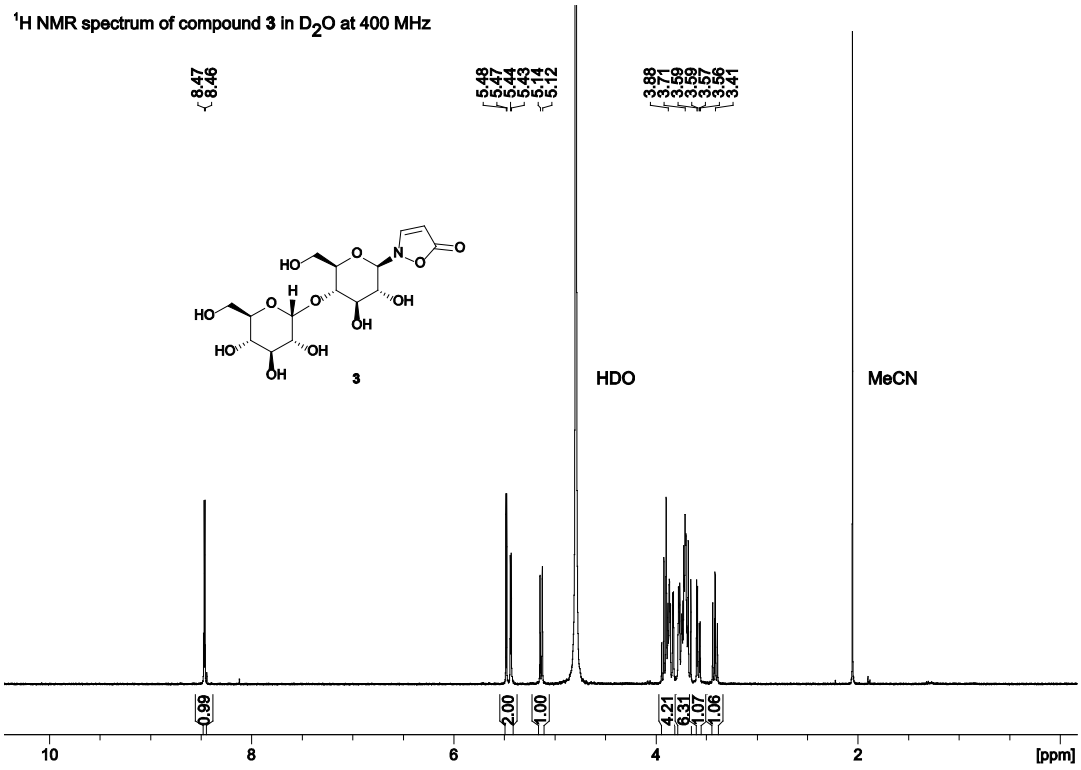


2

^{13}C NMR spectrum of compound **2** in D_2O at 100 MHz

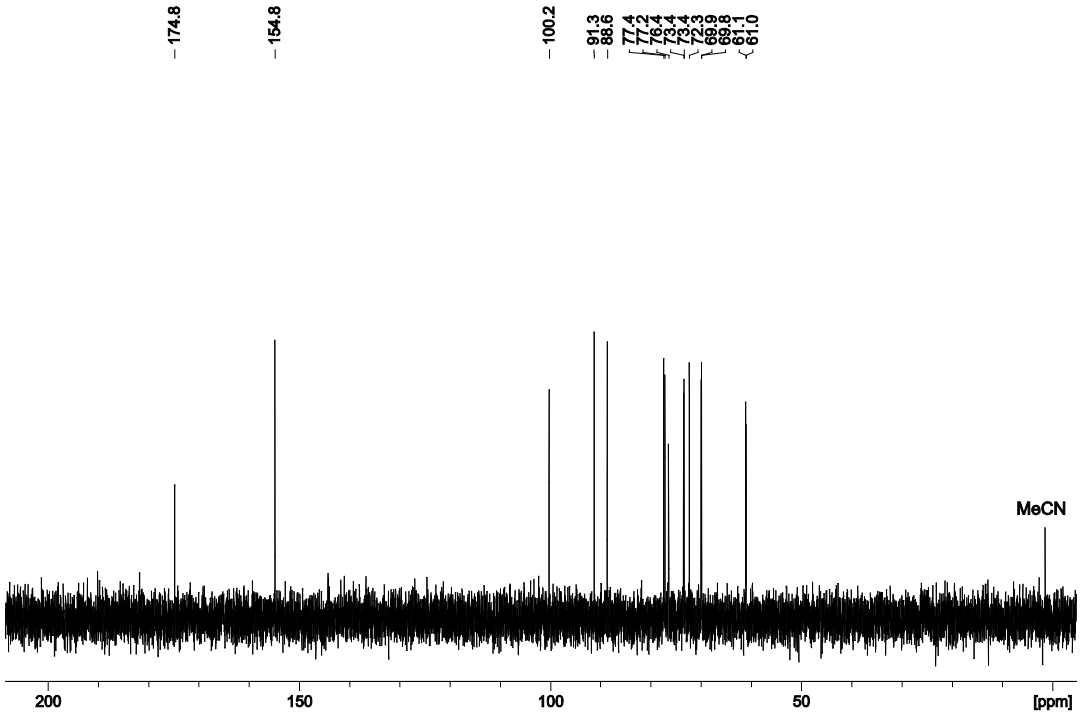


^{1}H NMR spectrum of compound **3** in D_2O at 400 MHz

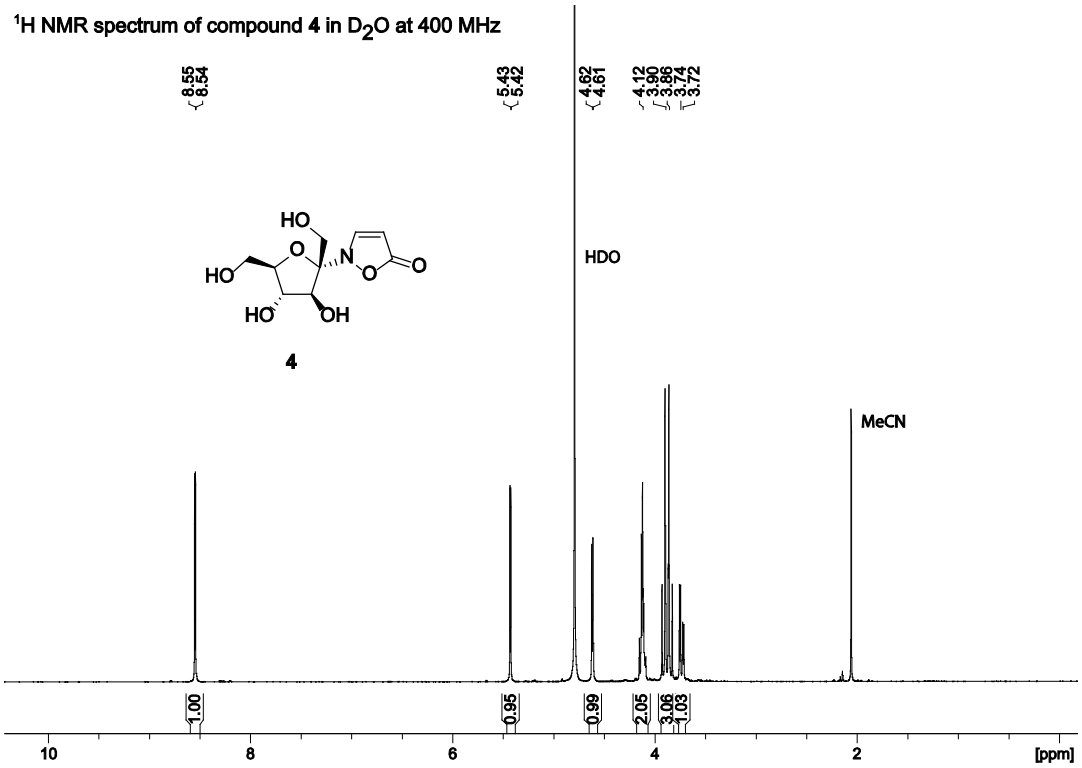


3

^{13}C NMR spectrum of compound **3** in D_2O at 100 MHz



^{1}H NMR spectrum of compound **4** in D_2O at 400 MHz



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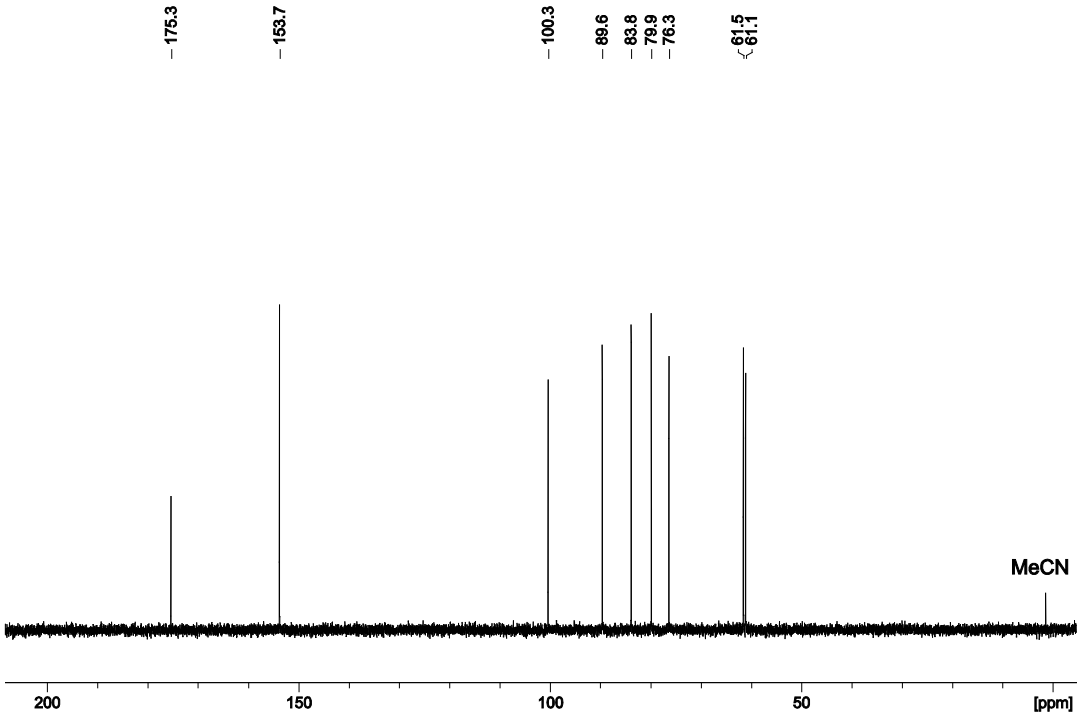
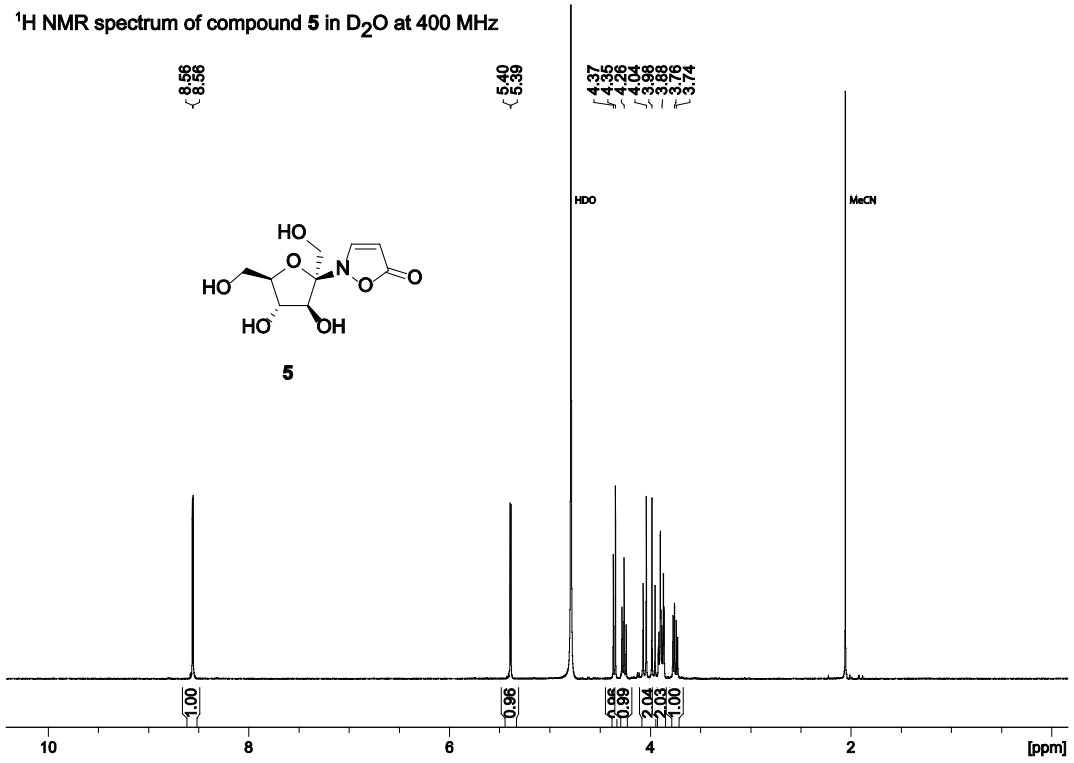
^{13}C NMR spectrum of compound **4** in D_2O at 100 MHz

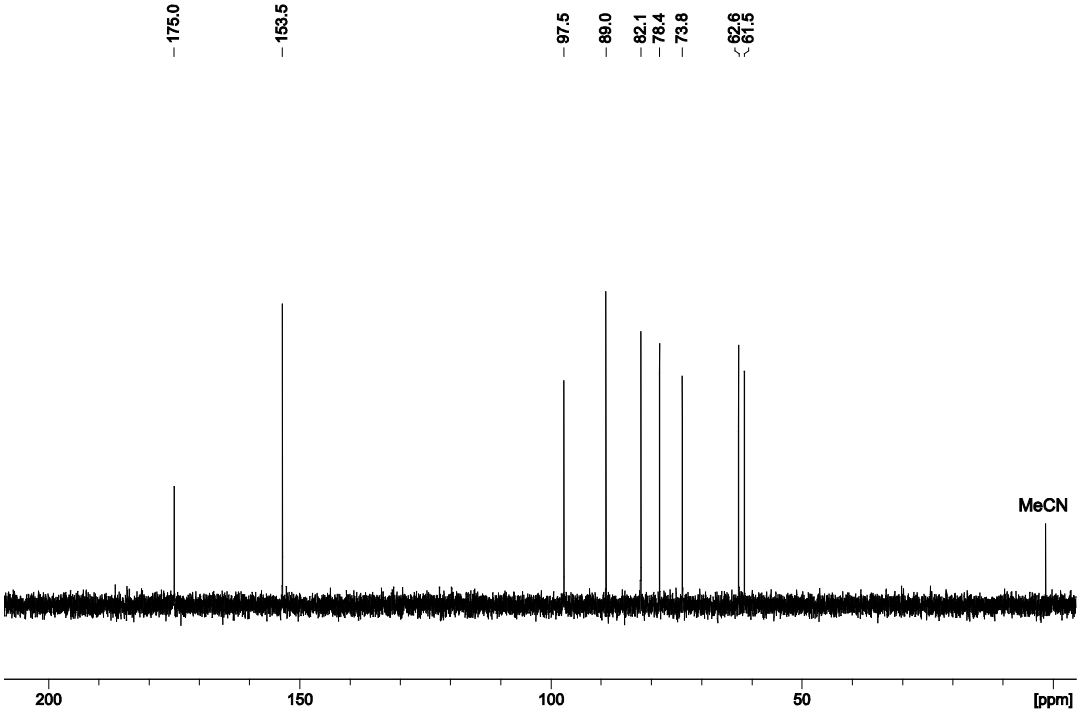


^{1}H NMR spectrum of compound **5** in D_2O at 400 MHz



5

^{13}C NMR spectrum of compound **5** in D_2O at 100 MHz



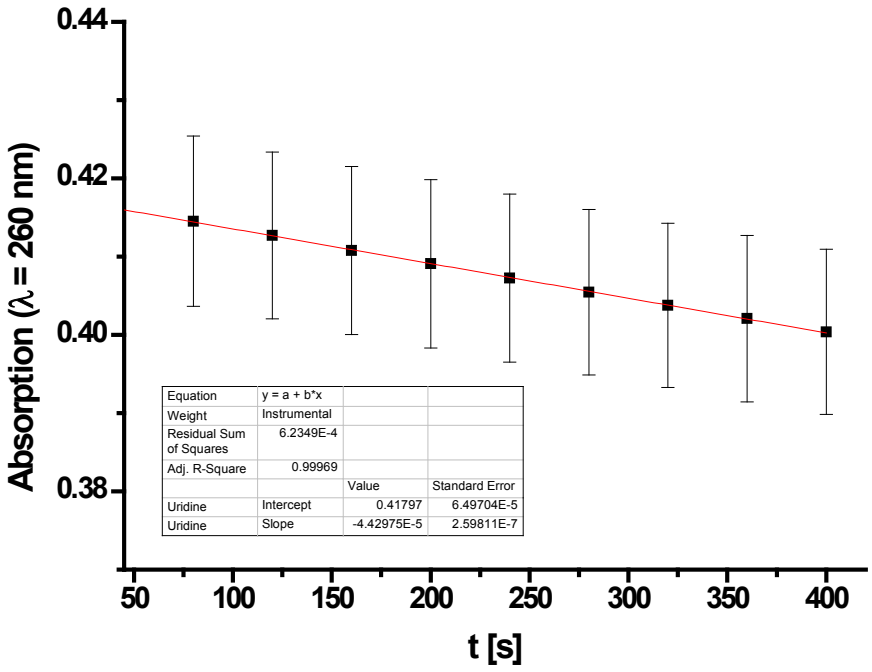


Fig. S1: Decay curve of uridine in Na_2HPO_4/NaH_2PO_4; λ_{max} = 254 nm; I_{261} = 0.18 mW/cm^2; pH = 7; d_{lamp} = 5 cm; rt; the error bars show the standard deviation (n = 5).

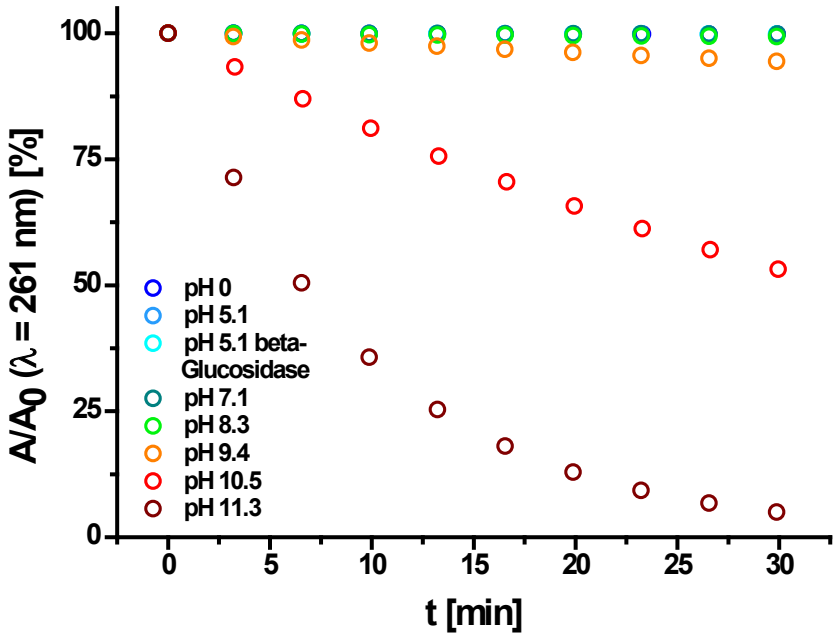


Fig. S2: Absorption of compound **1** at 261 nm under different pH conditions over the time.

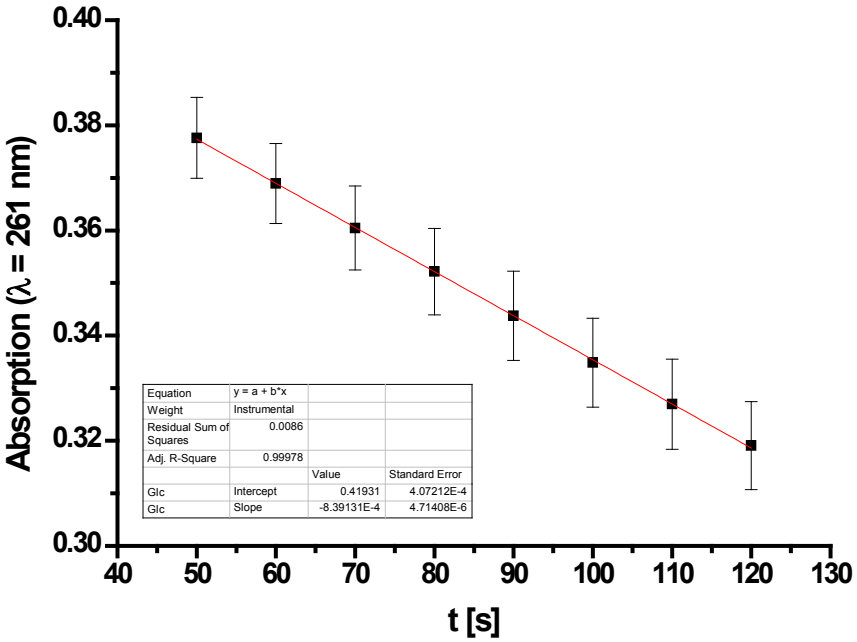


Fig. S3: Decay curve of comp. **1** in Na_2HPO_4/NaH_2PO_4; λ_{max} = 254 nm; I_{261} = 0.18 mW/cm^2; pH = 7; d_{lamp} = 5 cm; rt; the error bars show the standard deviation (n = 5).

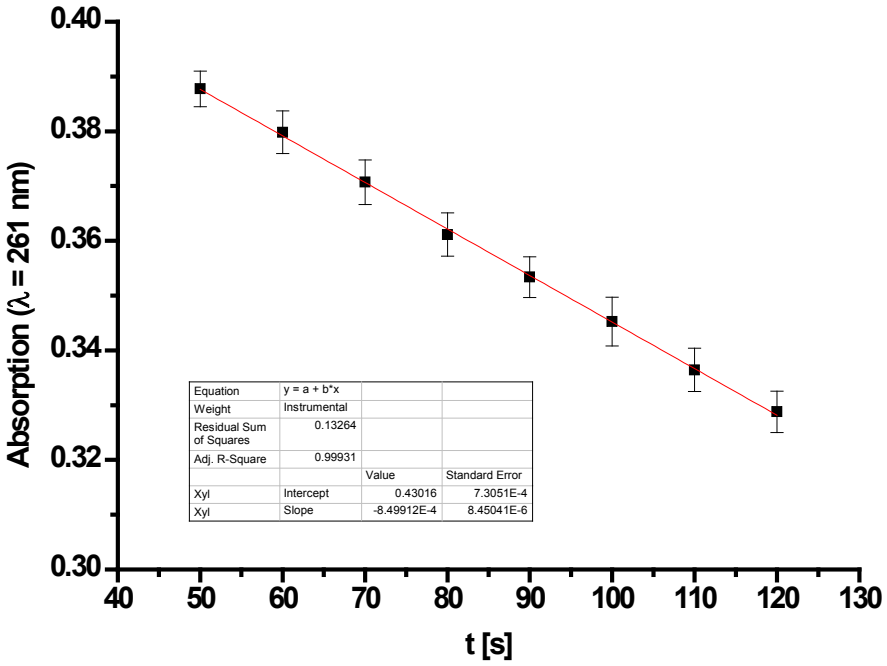


Fig. S4: Decay curve of comp. **2** in Na_2HPO_4/NaH_2PO_4; λ_{max} = 254 nm; I_{261} = 0.18 mW/cm^2; pH = 7; d_{lamp} = 5 cm; rt; the error bars show the standard deviation (n = 5).

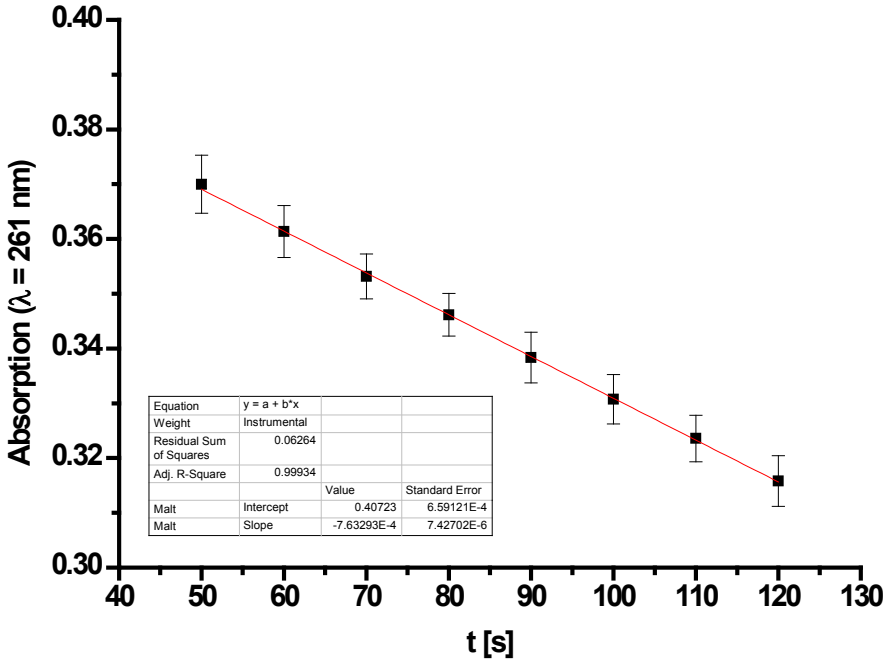


Fig. S5: Decay curve of comp. **3** in Na_2HPO_4/NaH_2PO_4; λ_{max} = 254 nm; I_{261} = 0.18 mW/cm^2; pH = 7; d_{lamp} = 5 cm; rt; the error bars show the standard deviation (n = 5).

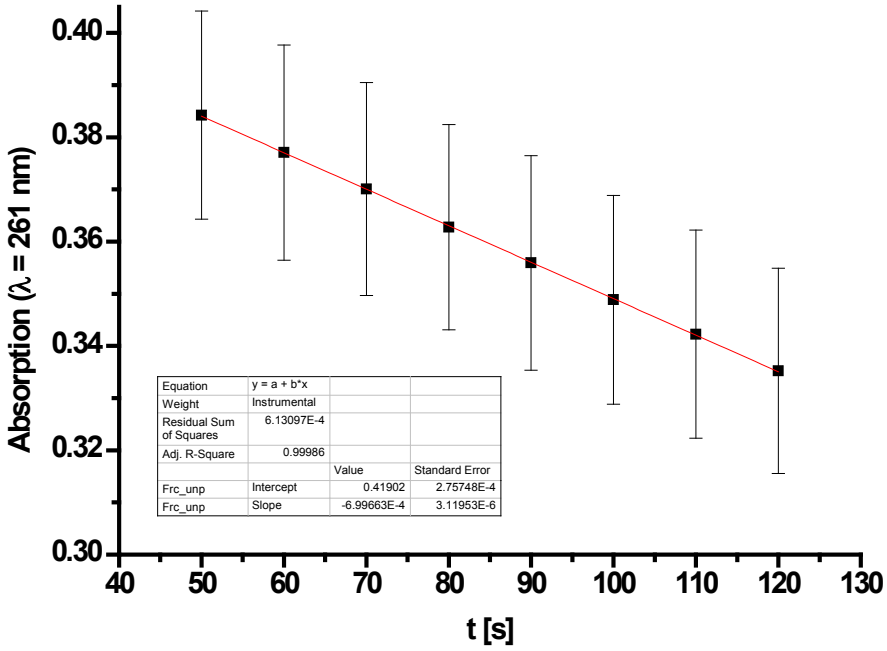


Fig. S6: Decay curve of comp. **4** in Na_2HPO_4/NaH_2PO_4; λ_{max} = 254 nm; I_{261} = 0.18 mW/cm^2; pH = 7; d_{lamp} = 5 cm; rt; the error bars show the standard deviation (n = 5).

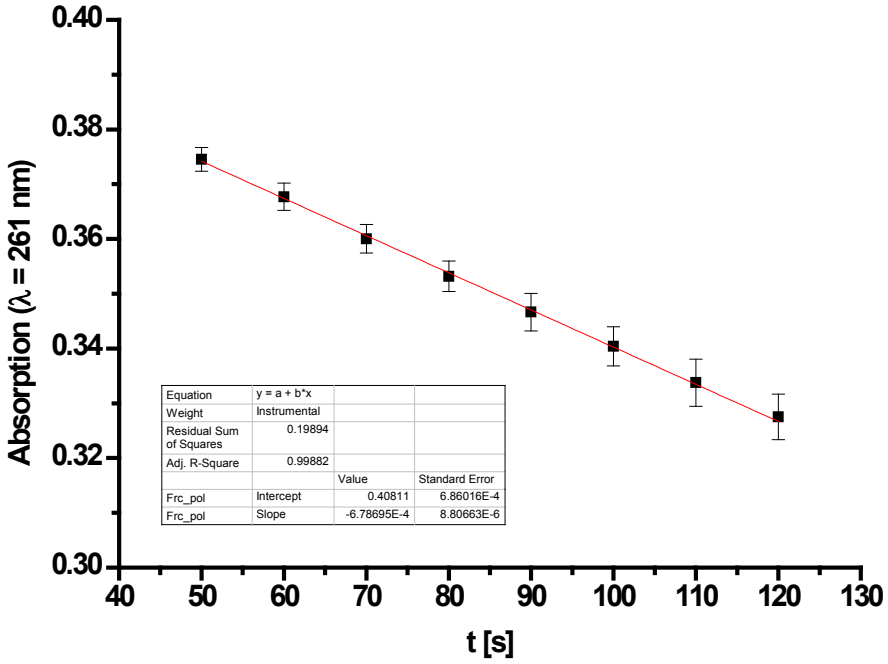


Fig. S7: Decay curve of comp. **5** in Na_2HPO_4/NaH_2PO_4; λ_{max} = 254 nm; I_{261} = 0.18 mW/cm^2; pH = 7; d_{lamp} = 5 cm; rt; the error bars show the standard deviation (n = 5).

8.4. Manuscript 3

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The subsequent manuscript has been used in the PhD thesis of Gerhard Pauls as a major part of this thesis, describing the analytical as well as taxonomic and ecological aspects of the novel findings.

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Two Defensive Lines in Juvenile Leaf Beetles; Esters of 3-Nitropropionic Acid in the Hemolymph and Aposematic Warning

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Abstract Juveniles of the leaf beetles in subtribe Chrysomelina have efficient defense strategies against predators. When disturbed, they transiently expose volatile deterrents in large droplets from nine pairs of defensive glands on their back. Here, we report on an additional line of defense consisting of the non-volatile isoxazolin-5-one glucoside and its 3-nitropropanoyl ester in the larval hemolymph. Because isoxazolin-5-one derivatives were not detectable in related leaf beetle taxa, they serve as a diagnostic marker for the Chrysomelina subtribe. Conjugation of isotopically labelled 3-nitropropionic acid to isoxazolin-5-one glucoside *in vivo* demonstrates its function as a carrier for the 3-nitropropanoyl esters. The previous identification of characteristic glucosides as precursors of the volatile deterrents underlines the general importance of glucosides for sequestration from food plants, and the subsequent transport in the hemolymph to the defense system. The combination of repellent volatiles with non-volatile toxic compounds in the hemolymph has the potential to create synergistic effects since the odorant stimulus may help predators learn to avoid some foods. The combination of the two defense lines has the advantage, that the hemolymph toxins provide reliable and durable protection, while the repellents may vary after a host plant change.

Keywords Chrysomelidae · Chemical defense · 3-nitropropionate esters · Isoxazolin-5-one glucoside · Aposematic warning

Introduction

Toxins are the most effective players on our planet when it comes to the manifold interactions in trophic networks. Reflecting successful relationships with their hosts, leaf beetles (family Chrysomelidae) of the taxon Chrysomelina have ingenious strategies that disarm plant toxins and, at the same time, produce a chemical defense against natural enemies. This chemical defense not only protects all developmental stages from larvae to adults, but also changes its composition during the life history of Chrysomelina beetles.

Adult beetles store and release defensive secretions from pronotal and elytral exocrine glands upon disturbance (Deroe and Pasteels 1982; Pasteels *et al.* 1989). The major components are isoxazolin-5-one glucosides esterified with up to three 3-nitropropionic acid (3-NPA) moieties (Pasteels *et al.* 2003; Sugeno and Matsuda 2002). As these compounds are originally not present in any of the beetle host plants, they are postulated to be synthesised by the insects themselves *via* pathways that remain to be explored (Randoux *et al.* 1991).

While the mode of action of the isoxazolinone moiety is unknown, 3-NPA is a naturally occurring neurotoxin that, when ingested, causes poisoning in both humans and domestic livestock by irreversibly inhibiting the mitochondrial succinate dehydrogenase (E.C. 1.3.5.1), a key enzyme of the

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citric acid cycle (Anderson *et al.* 2005; Beal *et al.* 1993; Huang *et al.* 2006). 3-Nitropropionic acid and its glucose esters have been identified in many members of the legume plant family (Fabaceae) and in certain fungi as a defense against herbivores (Chomcheon *et al.* 2005; Francis *et al.* 2013; Parry *et al.* 2011). Some insect herbivores can detoxify 3-NPA (Johnson *et al.* 2001; Majak *et al.* 1998; Novoselov *et al.* 2015). To date, Chrysomelina leaf beetles are the only insects in which 3-NPA and its derivatives have been described as allomones. The amounts of 3-NPA that predators of leaf beetles, for example ants or birds can tolerate before avoiding further consumption, remain to be determined.

Unlike adults, juveniles rely on volatile repellents whose molecular structure is entirely different. By displaying droplets of defensive secretions from nine pairs of everting glandular reservoirs located on their backs, the larvae have an extraordinary defense mechanism unparalleled in the insect world. The defensive chemicals in the Chrysomelina larval exudates are composed of four compound classes (Fig. 1): iridoids (cyclopentanoid monoterpenoids, *e.g.*, chrysomelidial **2**), aldehydes (salicylaldehyde **4** and benzaldehyde), esters (*e.g.*, phenethyl esters), and the naphtoquinone juglone (Hilker and Schulz 1994; Laurent *et al.* 2005; Matsuda and Sugawara 1980; Pasteels *et al.* 1986). Phylogenetic analysis of Chrysomelina species revealed that the composition of their secretions reflects a step-wise scenario of host-plant adaptation (Termonia *et al.* 2001).

The plant-independent biosynthesis of iridoids predated the sequestration of salicin, a plant-derived precursor from Salicaceae, used to produce the repellent salicylaldehyde (Kuhn *et al.* 2004; Pasteels *et al.* 1983b). Later in Chrysomelina beetle evolution, a sequestering Chrysomelina lineage – namely, the *interrupta* group – escaped the constraints of their host plant (willow) by shifting to birch. Due to the different secondary metabolites present in the two hosts,

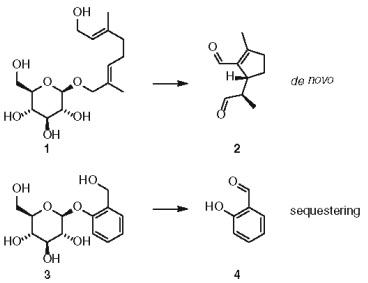

Fig. 1 Compounds implicated in the volatile chemical defense of the Chrysomelina larvae. 8-hydroxygeraniol glucoside (**1**) and salicin (**3**) are precursors of chryomelidial (**2**) and salicylaldehyde (**4**) in *Phaedon cochleariae* and *Chrysomela populi*, respectively

this shift resulted in modified larval exudates produced from the sequestered precursors. For example, the willow-feeding population of the species, *Chrysomela lapponica,* produces predominantly salicylaldehyde from sequestered salicin, whereas the birch-feeding population is able to take up a wide variety of glucosidically bound leaf alcohols. These leaf alcohols are further esterified with butyric acid, resulting in a cocktail of at least 60 esters in the defensive exudate (Geiselhardt *et al.* 2015; Termonia *et al.* 2001). Regardless of the different composition and origin of the defensive metabolites in the secretions of Chrysomelina larvae (*de novo vs.* sequestration), the synthesis of all Chrysomelina allomones includes glucoside intermediates (Discher *et al.* 2009).

Owing to their defensive volatiles, the larvae are protected against microbial infestation (Gross *et al.* 1998, 2002, 2008; Gross and Schmidtberg 2009; Gross *et al.* 2008), generalist arthropod predators (Blum *et al.* 1978; Hilker and Schulz 1994; Palokangas and Neuvonen 1992; Pasteels *et al.* 1983a, b), and insectivorous birds (Topp 1997). These non-specific volatile irritants, however, act as repellents rather than as toxins that target specific physiological processes (Pasteels *et al.* 1983a), and their ecological significance has to date remained poorly understood. As toxicity is often associated with warning signals such as colors, sound, taste, or odors (Pasteels *et al.* 1983a), we hypothesized that the volatile irritants also may be linked with toxins not yet identified in Chrysomelina larvae. Since the hemolymph provides the storage site for toxins in a wide range of insect species (Laurent *et al.* 2005; Opitz and Muller 2009), we analyzed the inventory of secondary metabolites in the hemolymph of juvenile chrysomelids by LC-MS and NMR.

Here, we report on the identification of isoxazolin-5-one glucoside and its 6-nitropropanoate in the larval hemolymph of all tested Chrysomelina species. Previously, these compounds had been attributed exclusively to the adults. However, this finding leads to the conclusion that Chrysomelina species are protected by isoxazolinone glucosides and their 3-NPA esters throughout the beetle life history. Hence, in addition to the defensive odor released from their dorsal glands, the larvae possess toxins in their hemolymph. This association may contribute synergistically to protection against an array of vertebrate and invertebrate enemies. Further, we detected glucoside precursors for the volatile secretions in the larval hemolymph, a discovery that underlines the importance of sugar derivatives as carriers for controlled translocation processes and for preventing the insects from self-poisoning.

Methods and Materials

Insect Rearing *Chrysomela populi* (L.) was collected near Dornburg, Germany on *Populus maximowiczii* × *Populus*

nigra. Beetles were propagated using a cycle of 16 h L and 8 h D at 18±2 °C in light and 13±2 °C in darkness. *Phaedon cochleariae* (F.) was collected from Brassicaceae close to the city of Bayreuth (Germany) and kept as a continuous culture in the laboratory (Discher *et al.* 2009). Larvae were reared on *Brassica rapa* subsp. *pekinensis* "Cantonner Witkrop" (Quedlinburger Saatgut, Quedlinburg, Germany) in a Snijder chamber (Snijders Scientific, Tilburg, Netherlands) in a cycle of 16 h L / 8 h D and 13 °C/11 °C±1 °C. The low temperature (13 °C) was necessary to reduce fungal growth on the food plant. Willow feeding *C. lapponica* (L.) were collected in the Altai Mountains in East Kazakhstan, near Katon-Karagai in the Katon-Karagaisky State National Nature Park (2100 m altitude). Birch-feeding *C. lapponica* was collected from *Betula rotundifolia* in the Altai Mountains in East Kazakhstan, close to Uryl, near the Burkhat Pass in the Katon-Karagaisky State National Nature Park (2130 m altitude). All other species were collected in the field, see Table S1 for details.

Preparation of Samples from the Hemolymph, Frass, and Whole Larvae Extracts Hemolymph samples were collected as described previously (Bodemann *et al.* 2012) in capillaries that were sealed immediately after collection and stored at −20 °C until use. Hemolymph weight was determined by measuring the weight of a filled capillary minus its dry empty weight (Mettler-Toledo XS 205, Greifensee, Switzerland). For LC-MS measurements, the hemolymph was diluted with 50 % aqueous MeOH in a ratio of 1 μl hemolymph per 100 μl solvent. Frass samples of *P. cochleariae* (2.5 mg), *C. populi* (13 mg), and *C. lapponica* (5 mg) were extracted with water and analyzed by LC-MS.

For crude extracts prepared from complete larvae, each larva was weighed individually using an ultra-microbalance (XS205; $d = 0.01$ mg; Mettler-Toledo, Greifensee, Switzerland). Individual larvae were frozen separately in liquid N_2 and macerated in 500 μl MeCN using a Geno grinder. After centrifugation (10,621 rpm, 10 min, room temperature), the supernatant was subject to LC-MS analysis.

Analysis of Glucosides by LC–MS Analyses were carried out using an Agilent HP1100 HPLC system equipped with an RP-C18 column, LiChroCART (250×4 mm, 5 μm;Merck KGaA, 64271, Darmstadt, Germany) connected to a Finnigan LTQ (Thermo Electron Corp, Dreieich, Germany) operated in the APCI mode (vaporizer temperature: 500 °C, capillary temperature 300 °C). Standard compounds for identification were either purchased (Sigma-Aldrich (St. Louis, MO, USA) or synthesised. Isoxazolin-5-one glucoside and its esters were synthesised according to previously described protocols (Becker *et al.* 2013, 2015).

Samples were analyzed by injection (5 μl) and by the application of a gradient elution. The following protocol was used: 100 % solvent A (H_2O+0.1 % HCOOH) and 0 % solvent B (MeCN+0.1 % HCOOH), linear gradient to 60 % solvent B in 35 min. Extract samples of whole larvae were analyzed by injecting a 5 μl sample and using an isocratic elution with 35 % solvent B (*v/v*) in H_2O +0.1 % HCOOH. For identification and quantification, the formic acid adducts $[M+HCOOH-H]^-$ were used (*m/z* 292 for 2-(β-D-glucopyranosyl)-3-isoxazolin-5-one (**5**), *m/z* 393 for 2-[6′-(3″-nitropropanoyl)-β-D-glucopyranosyl]-3-isoxazolin-5-one (**6**), *m/z* 331for salicin (**3**), and *m/z* 377 for 8-hydroxygeraniol-8-O-*β*-D-glucoside (**1**).

Analysis of Crude Larval Hemolymph by NMR Hemolymph from 20 larvae of *P. cochleariae* or *C. populi* was collected and taken up in 200 μl CD_3OD for $^1H/^2D$-exchange. The solution was concentrated under reduced pressure and dissolved in 500 μl CD_3OD. One-dimensional ^1H NMR spectra were recorded on a Bruker AV400 using water suppression (purge). Two-dimensional double quantum-filtered (*dqf*)-COSY spectra with phase cycling were recorded on a Bruker AV400. A total of 32 scans were acquired using a time domain of 8 k in F2 (acquisition time of 1.2 s) and 512 increment in F1. Spectra were zero-filled to 8 k × 4 k prior to Fourier transformation and phasing using the Topspin software (Bruker). Heteronuclear HSQC and HMBC spectra were recorded using Bruker AMX500 with a cryoprobe. Samples were dissolved in 100 μl CD3OD using 2 mm NMR vials. For HSQC spectra, 40 scans were acquired using a time domain of 1 k in F2 and 256 increments in F1. For HMBC spectra, 256 scans were acquired using a time domain of 4 k in F2 and 128 increments in F1. Spectra were zero-filled to 4 k × 2 k prior to Fourier transformation and phasing using the Topspin software (Bruker).

Statistical Analysis Linear regressions were used to investigate whether the amount of **5** and **6** changed with the weight of the larvae. In order to achieve homogeneous variances and normality of the residuals, data were square root transformed. Data were analyzed with SigmaPlot 11.0.

Synthesis of Labelled [1-^{13}C, 3-^{15}N]-3- Nitropropionic Acid and Injection of Labelled 3-NPA into the Larval Hemolymph Stable isotope labelled [1-^{13}C,3-^{15}N]-3-nitropropinoic acid was synthesized according to Baxter *et al.* (1992) by using $K^{13}CN$ instead of $Na^{13}CN$, and $Na^{15}NO_2$ instead of $NaNO_2$.

The mass of third-instars (15 days after hatching) was measured on an ultra-microbalance (Mettler-Toledo, Greifensee, Switzerland). Ice-chilled larvae were injected dorso-medianally in the intersegmental membrane behind the pronotum using a pulled glass capillary as a needle connected to a nanoliter-injection pump (WPI, Sarasota, FL, USA), mounted on a three-axis-micromanipulator. For labelling

experiments, each larva was injected with 200 ng 3-NPA per mg body weight in 122 nl injection-buffer (Bodemann *et al.* 2012), representing a sublethal dose determined previously in a pilot experiment. To test larval tolerance to 3-NPA, 10 *P. cochleariae* larvae each were injected with the following concentrations: 100, 200, 300, 400 ng/mg body weight. While 300 ng/mg body weight was tolerated (10 of 10 survived), injection of 400 ng/mg body weight was fatal (10 of 10 died).

Results

Identification of Isoxazolinone Glucosides in the Larval Hemolymph of Leaf Beetles To detect isoxazolinone glucosides in leaf beetle hemolymph, the following model species were analyzed: *P. cochleariae,* representing the iridoid *de novo* producers; *C. populi* and willow-feeding *C. lapponica,* representing the salicin-sequestering species. The larval hemolymph samples were analyzed by HPLC-MS. APCI ionization was chosen because it is less susceptible to matrix effects (Peters and Remane 2012). Compounds were identified by comparing HPLC-MS chromatograms of the natural samples with the spectra of synthetic standards (Fig. 2, see Fig. S1 and S2 for mass spectra).

To confirm the identity of the presumed hemolymph toxins and to obtain an unbiased assessment of the whole metabolome, we employed NMR spectroscopy. Previous reports demonstrated that ^{1}H NMR spectroscopy is well suited to analyze complex metabolome mixtures, including insect hemolymph samples (Lenz *et al.* 2001; Phalaraksh *et al.* 1999; Poynton *et al.* 2011). For our analysis of the crude unfractionated *C. populi* and *P. chochleariae* hemolymph, we used the two-dimensional double quantum-filtered correlation spectroscopy (*dqf*-COSY), which provides outstanding sensitivity and dynamic range along with a wealth of structural information. Analysis of the *dqf*-COSY spectra (Fig. S3) indicated the presence of several free amino acids such as alanine, valine, leucine, isoleucine, threonine, and proline (Table S2), along with characteristic signals corresponding to two dominant β-glucosidic components. Their heteroaromatic aglycone moieties were identified as isoxazolinones based on two characteristic AX-spin systems at δ_H 8.453 and 5.315 or at δ_H 8.433 and 5.344 that both displayed a coupling constant of ^{3}J=3.7 Hz. The linkage of the glucose and isoxazolinone moieties was established by complementary HSQC and HMBC correlations from the anomeric hydrogen to the β-carbon. The β-configuration of the glycosidic bond was deduced from the vicinal coupling constant $^{3}J_{1',2'}$=9.2 Hz for the anomeric hydrogen. Both isoxazolinone glucosides differed in the chemical shifts of the 6′-position, indicating 6′-acylation in one of the two components. This assumption was confirmed by HMBC correlations from the 6′-methylene protons to a carbonyl moiety at δ_C 171.5 ppm. Furthermore, this carbonyl group displayed additional HMBC correlations to an A_2M_2 spin system at δ_H 3.02 δ_C 31.7 ppm and δ_H 4.71 δ_C 70.7 ppm, indicating a 3-nitropropanoate substitution. Comparison of the ^{1}H and ^{13}C NMR data with data of the authentic standard obtained by synthesis as previously described (Becker *et al.* 2013, 2015) confirmed our structure assignment. Since the α- and β-anomers of compound **5** can be separated easily by LC (RP-C18 column, separation factor R=1.74 (Becker *et al.* 2013, 2015) by using isocratic elution with acetonitrile and water (3:97, v:v), the larval defense compound consists of the pure β-anomer (>99 %).

Both components, isoxazolinone β-glucoside and its 6′-nitropropanoate (Fig. 2, Figs. S1, S2), have been previously described in eggs and adults of the subtribe Chrysomelina (Matsuda and Sugawara 1980; Pasteels *et al.* 1986), but the presence of these compounds in the larval hemolymph previously was unknown.

Occurrence of Isoxazolin-5-one Derivatives in Various Chrysomelidae To determine the distribution of isoxazolin-5-one derivatives in the leaf beetle family, the larval hemolymph of Chrysomelina beetles and related subtribes Chrysolinina and Galerucinae were analyzed. In all tested species of Chrysomelina, the isoxazolinone glucoside **5** could be quantified, whereas in the two other subtribes, the glucoside was not detectable (Fig. 3, Table S3). The corresponding nitropropanoyl ester **6** was detectable in the Chrysomelina hemolymph samples of the *de novo* iridoid-producing species *P. cochleariae*, in the salicin-sequestering species *C. populi*, *Chrysomela saliceti, Chrysomela tremulae*, and willow-feeding *C. lapponica* and in the ester-producing *C. lapponica* which feeds on birch (Fig. 3). A limiting factor is the lack of the stability of the 3-NPA esters in the hemolymph, while the isoxazolinone glucoside **5** exhibits as an *N*-glucoside with exceptional stability. Even in acidic media, the compound is stable (Becker *et al.* 2015). Hence, notwithstanding the production strategy of volatile deterrents in the defensive glands of juvenile Chrysomelina, all tested Chrysolinina species posess a hemolymph-based chemical defense. Leaf beetle taxa other than Chrysomelina do not possess any of the isoxazolin-5-one derivatives.

Isoxazolin-5-one Derivatives are Produced During Larval Development Endogenously Considering the high concentrations of compounds **5** and **6** in the larval hemolymph and results reported by (Randoux *et al.* 1991), who describe the *de novo* production of isoxazolin-5-one derivatives in adult chrysomelids, we asked whether the larvae also are able to synthesize these derivatives endogenously during the juvenile stage. Therefore, we analyzed these compounds in different larval stages in *P. cochleariae* as representatives. Whole larvae extracts were analyzed by LC-MS, and the amount of

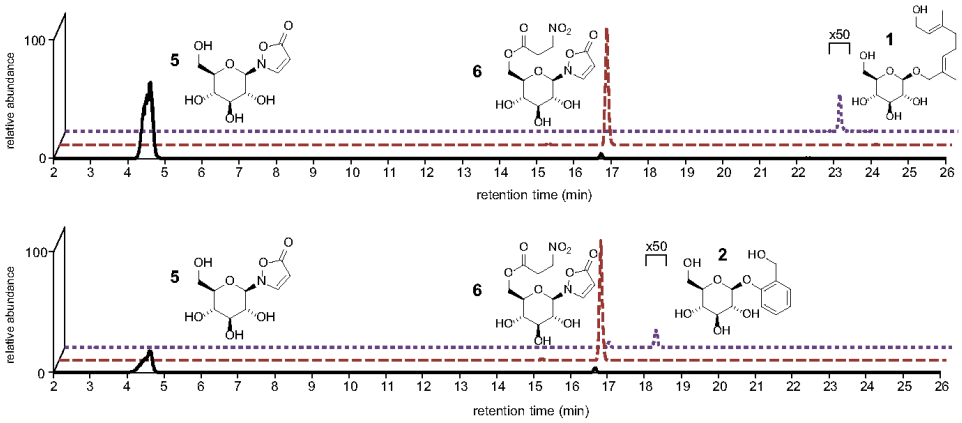

Fig. 2 Liquid chromatograms of larval hemolymph of chrysomelina leaf beetles. From top to *bottom*: *Chrysomela populi*, *Phaedon cochleariae*. Traces for formic acid adducts $[M+HCOOH]^-$ for glucosides are shown: isoxazoline-5-one-glucoside (*m/z* 292, solid line), isoxazoline-5-one-glucoside 3-NPA ester (*m/z* 393, *dotted* line), salicin (*m/z* 331, dashed line), 8-hydroxygeraniol glucoside (*m/z* 377, *dashed line*)

isoxazolinone glucoside **5** and its 3-NPA ester **6** in nmol/mg plotted *vs.* fresh body weight (ranging from 2 to 15 mg) as shown in Fig. S4. We found that the concentration of compound **5** (in nmol/mg body weight) remained constant (regression analysis, $P=0.448$) in larvae of different body weight, whereas the concentration of the glucoside ester **6** increased with body weight ($P<0.001$), indicating that an increasing amount of total isoxazolin-5-one derivatives must be produced during larval development.

In order to find out if the toxin level in the hemolymph can be regulated by excretion *via* the malpighian tubules or hindgut, we analyzed the frass of chrysomeline larvae. However, we could detect neither the glucoside **5** nor the ester **6** in the frass of *P. cochleariae*, *C. populi*, or willow-feeding *C. lapponica* within the detection limits. Both analytes also were not detectable in defensive secretions of the larvae. This suggests that a strongly limited excretion of isoxazolinone derivatives contributes to their accumulation in the hemolymph, which also requires an internal regulation of the ratio of glucoside **5** to ester **6**.

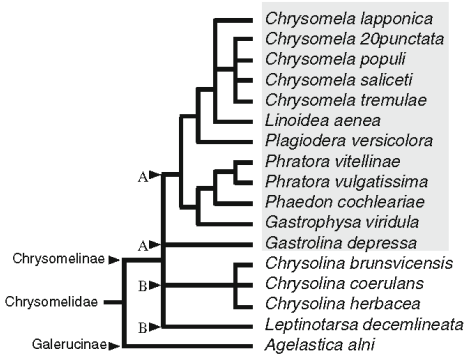

Fig. 3 Phylogeny in relation to the presence of isoxazolinone glucoside and its 6-nitropropanoate ester in the hemolymph. The phylogeny is adapted from Termonia and Pasteels (1999), Gomez-Zurita *et al.* (2007), and Daccordi (1994) to represent different Chrysomelidae species in relation to the presence of isoxazolinone glucoside and 6-nitropropanoate ester in the larval haemolymph (marked with *grey box*). The branch points marked with A show the separation into the tribe Chrysomelini (subtribe Chrysomelina) and B into the tribe Chrysolinini (subtribe Doryphorina)

Free 3-NPA is Conjugated to Isoxazolin-5-one Glucoside 5

Given the toxicity of free 3-NPA, we tested the metabolic ability of Chrysomelina larvae to accept free 3-NPA as a substrate for the biosynthesis of the ester compound **6**. For this purpose, sublethal doses (200 ng/mg larva) of stable isotope labelled 3-NPA ($[^{15}N, 1\text{-}^{13}C]$ 3-NPA) were injected into the larval hemolymph of *P. cochleariae*, *C. populi*, and willow-feeding *C. lapponica*. HPLC-MS analyses revealed the incorporation of 3-NPA into isoxazolin-5-one glucoside **5**, forming the corresponding ester **6**. Diesters or triesters, as reported in adult leaf beetle secretions (Matsuda and Sugawara 1980), were absent. Furthermore, the enrichment of the isotope signals at $[M+2]$ in the hemolymph compared to buffer-treated control groups was determined. The values ranged between 7 and 24.5 %, indicating a tolerance to free 3-NPA (Fig. S5). The isotope enrichment for compound **6** in *C. populi* was 13.2 %±4.3 % (arithmetic mean±standard deviation, $N=4$

for each species), in the case of *P. cochleariae* it was 7 %±1.3 %, while *C. lapponica* showed 24.5 %±9.5 % enrichment. In summary, 3-NPA is esterified to **6** with differences in efficiency depending on the examined species. Compound **5** apparently serves as a carrier to attach free 3-NPA to form the non-toxic ester **6**.

Detection of Salicin and 8-Hydroxygeraniol-8-O-ß-D-glucoside in the Larval Hemolymph In addition to examining the hemolymph production of isoxazolin-5-one derivatives, we screened for *de novo*-produced as well as sequestered precursor glucosides of the volatile deterrents in the hemolymph (Fig. 1). Under the chosen chromatographic conditions, our target compounds showed signals at m/z=377 $[M+HCOO]^-$ for 8-hydroxygeraniol glucoside **3** in *P. cochleariae* as well as m/z=331 $[M+HCOO]^-$ for salicin **1** in *C. populi*. The compounds were identified by comparison of HPLC-MS chromatograms of the natural samples with the spectra of commercially available standards. To confirm our identification of previously mentioned glucosides in the hemolymph of *P. cochleariae* and *C. populi,* we reanalyzed the *dqf*-COSY spectra. These confirmed the presence of small amounts of 8-hydroxygeraniol glucoside **3** as well as salicin **1** (Fig. S6). In summary, we identified 8-hydroxygeraniol glucoside **3** and salicin **1** in the hemolymph of *P. cochleariae* and *C. populi*, respectively. This confirms a function of the hemolymph as a transport matrix for the isoxazoline-glucoside **5**, its 3-NPA ester **6**, and the deterrent precursors, produced *de novo* or sequestered, *en route* to the tissue of destination (Discher *et al.* 2009).

Discussion

The toxicity of insects often is linked to warning signals (Pasteels *et al.* 1983a). The adults of many Chrysomelina species, for example, have aposematic red elytra advertising the toxicity of 3-NPA esters of isoxazolinone glucosides and their break down product 3-NPA. Compared to adults, the larvae possess a strikingly different defense mechanism. When disturbed, they display large droplets that contain secretions from eighteen glands; these droplets change the larvae's appearance dramatically. As predators often are conservative when assessing the size of their prey, this behavior alone may prevent life-threatening attacks (Cohen *et al.* 1993). In addition to the appearance of the larvae, their odor also changes since the droplets contain volatile chemicals in high amounts, such as iridoids or salicylaldehyde. Besides their repellent effect on predators, these irritants have nonspecific toxic effects. Iridoids, for example, can bind proteins covalently that might have adverse effects upon ingestion (Kim *et al.* 2000), whereas salicylaldehyde exhibits a non-specific cytotoxic effects to insect cell cultures (Gross *et al.* 2002).

Our discovery of isoxazolinone-based hemolymph toxins led us to revise the view of the defense of Chrysomelina leaf beetles (Fig. 4). The 3-NPA ester **6** itself is a deterrent, as demonstrated with ants (Pasteels *et al.* 1986; Sugeno and Matsuda 2002). Furthermore, 3-NPA is a cytotoxin that interferes with mitochondrial respiration (Huang *et al.* 2006). Although adults possess esterase activity in their secretions, and thus, are able to cleave the esters of isoxazolin-5-one glucosides **5** to liberate 3-NPA, it is conceivable that the larvae have to be ingested to release toxic components by predator digestion.

The two described mechanisms of chemical defense, volatile and non-volatile compounds, could have synergistic effects. The odorant signal, *e.g.*, salicylaldehyde, could be a conditioning stimulus, linking the conspicuous odor to the hemolymph toxin. This system, known as olfactory aposematism (Weldon 2013), is effective mainly for vertebrate predators, such as birds; it is how they learn to avoid certain food. As tree-living *Chrysomela* species share their habitat with birds, the strongly odoriferous salicylaldehyde could be especially effective against this category of predators (Topp 1997). Based on the different allomones developed by Chrysomelina beetles, this taxon represents an unrivalled case study in chemical ecology, which illuminates the concerted action of diverse defense strategies during the adaptation of herbivorous insects to a given niche in an ecosystem.

Research into these hemolymph toxins extends our understanding of the chemical defense of chrysomeline leaf beetles considerably (Fig. 3). Sequestering leaf beetle larvae have

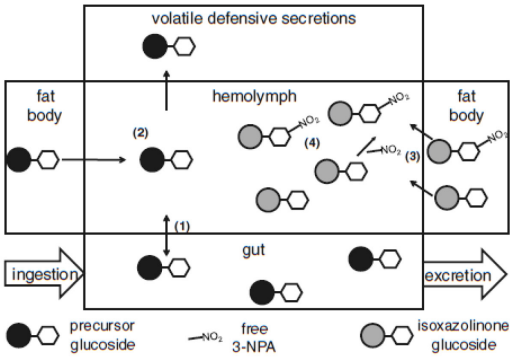

Fig. 4 Scheme of glucoside transport in leaf beetle larvae. Glucosides implicated in the volatile defense are ingested with the food. Transport proteins mediate the uptake of glucosides into the hemolymph (**1**). Precursor glucosides, either sequestered from food or synthesized autogenously, are selectively transported to the defensive glands for further processing (**2**). Isoxazolinone glucosides are produced in the fat body and released into the hemolymph (**3**). Free 3-NPA can be conjugated to isoxazolin-5-one glucoside to prevent autointoxication (**4**)

adapted to use plant-derived precursors to produce their defensive secretions, which has economic advantages but at the same time restricts host-plant affiliation. One Chrysomelina lineage, however, must have escaped the host-linked constraints (precursor uptake) by shifting host-plant families. Consequences for the changing composition of the secretions, for example, were seen in the different populations of the species *C. lapponica*, many of which shifted from salicin-rich willow species to salicin-poor or even salicin-devoid birch species. With the isoxazolinone-based defense, it becomes clear that the larvae are not as dependent on their volatile defense as has been previously suggested. Instead, the hemolymph toxins provide protection, independent from changes in the composition of the repellent secretions after a host-plant shift of a sequestering species such as *C. lapponica.*

Having detected isoaxazolinone glucoside **5** and its 3-NPA esters **6** in *C. lapponica*, *C. populi*, and *P. cochleariae*, we screened additional species of the Chrysomelidae family (see Fig. 3) for isoxazolinone derivatives in the larval hemolymph to obtain an estimate of the occurrence of these defensive compounds. Interestingly, although isoxazolinone glucoside **5** has been found in all analyzed members of the subtribe Chrysomelina, it has been detected neither in the larval hemolymph nor in adult secretions of species of the neighbouring subtribe Chrysolinina or in *Agelastica alni*, a member of the subfamily Galerucinae.

Consequently, the defense based on isoxazolinone derivatives throughout all developmental stages represents a trait unique to Chrysomelina beetles, and as such may be regarded as a chemomarker for this subtribe. For example, *G. depressa*, lately classified as a member of the subtribe Chrysomelina (Pasteels *et al.* 2003), also contains isoxazolinone glucoside **5**, which supports its classification into this taxon.

Considering the high concentrations of isoxazolinone glucosides **5** and **6** in the larval hemolymph, we asked whether juvenile Chrysomelina beetles derive these compounds from the eggs as a parental gift (Pasteels *et al.* 1986) or produce them *de novo* during larval development, as has been suggested for adult chrysomelids (Randoux *et al.* 1991). While an increase in ester compounds **6** during larval development was measured, the concentrations of the isoxazolinone glucoside **5** remained constant (Fig. S4). This suggests that these compounds are produced autogenously during larval development. As none of the respective host plants produce isoxazolinone derivatives, the most plausible scenario for their existence is autogenous synthesis. Furthermore, the increased overall concentration of isoxazolinone derivatives in the hemolymph can result from its lack of excretion by the malpighian tubules, as indicated by the complete absence of **5** and **6** in the larval frass of *P. cochleariae*, *C. populi*, and *C. lapponica*.

Neither compound is exported to the defensive system that encloses the isoazolinone glucosides **5** and **6** efficiently in the hemolymph. The biosynthesis of the isoxazolinone derivatives most likely starts from the metabolism of amino acids (Randoux *et al.* 1991), in particular β-alanine, which is efficiently incorporated into **6** (unpublished); however, neither the enzymatic steps of the pathway nor the regulation of the observed ratio of compound **5** to **6** have to date been resolved in the chrysomelids.

Autolysis of isoxazolinone glucoside ester **6** would lead to free 3-NPA and consequently to autointoxication. To address this possibility, stable isotope labelled 3-NPA was injected into Chrysomelina larvae. Labelled isoxazolinone glucoside ester **6** indicated the conjugation of free 3-NPA to the isoxazolinone glucoside **5**. No other detoxification strategies have been found in the Chrysomelina beetles. Such strategies have been reported from other organisms, including the oxidation of 3-NPA that was reported from microbes and plants (Francis *et al.* 2013), the conjugation to amino acids reported from *Spodoptora littoralis* (Novoselov *et al.* 2015) and melanopline grasshoppers (Johnson *et al.* 2001), or the formation of glucosides (miserotoxin) observed in grasshoppers (Johnson *et al.* 2001). Our findings have two consequences: first, the glucoside and free 3-NPA must be considered as the biosynthetic building blocks of the ester **6**; and second, isoxazolinone glucoside **5** likely serves as a storage site for the neurotoxin, which displays the physiological role of isoxazolinone glucoside **5** in Chrysomelina beetles.

The hemolymph surrounds all organs and is thus a vital transport medium between insect tissues. Intense research over the last decades has demonstrated the existence of a transport network in Chrysomelina larvae that is nonspecific in terms of import of dietary glucosides into the hemolymph (Discher *et al.* 2009; Strauss *et al.* 2013). While the larvae excrete glucosides that have not been utilized, they also transport genuine precursors into the defensive glands. In this study, we report for the first time the presence of actual *O*-β-D glucosides in the hemolymph of chrysomelid larvae, such as salicin **1** and 8-hydroxygeraniol-8-*O*-β-D-glucoside **3**, which support the transport model presented earlier. Our results lead us to conclude that the open circulation in the hemolymph of Chrysomelina larvae serves on the one hand as a transit site for the glucoside intermediates of the defensive secretions and on the other hand as a storage reservoir for the isoxazolinone derivatives.

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Author contributions G.P, S.H.vR, A.B., and W.B. designed research and analyzed the data, G.P. performed LC-MS analysis, T.B. synthesized authentic standards and isotope-labelled precursors, P.R. constructed the

phylogenetic tree, R.R.B. and J.P. provided reagents and research materials, C.P. and S.H.vR. performed NMR spectroscopic analysis, G.P., S.H.vR., A.B., and W.B. wrote the paper.

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TWO DEFENSIVE LINES IN JUVENILE LEAF BEETLES; ESTERS OF 3-NITROPROPIONIC ACID IN THE HEMOLYMPH AND APOSEMATIC WARNING

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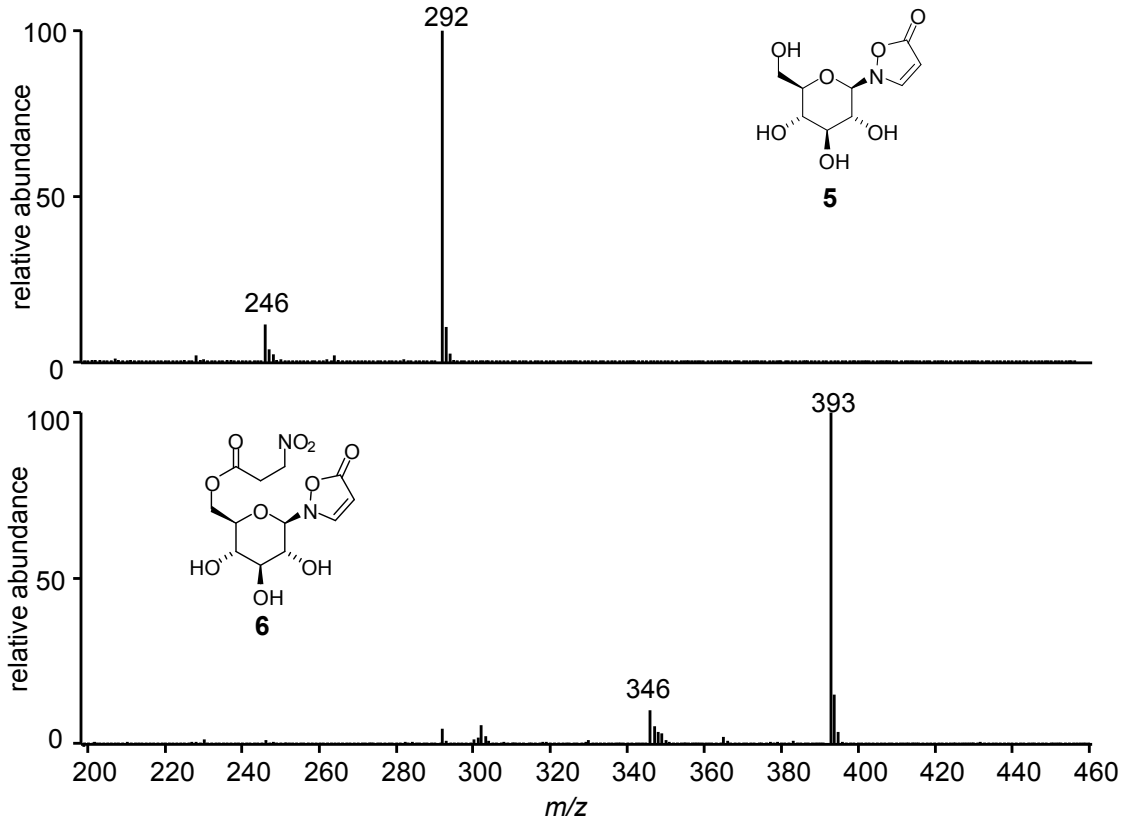

Fig. S1 Mass spectra of isoxazoline-5-one-glucoside and isoxazoline-5-one-glucoside ester. Marked signals correspond to $[M]^-$ and$[M+HCOOH]^-$ ions

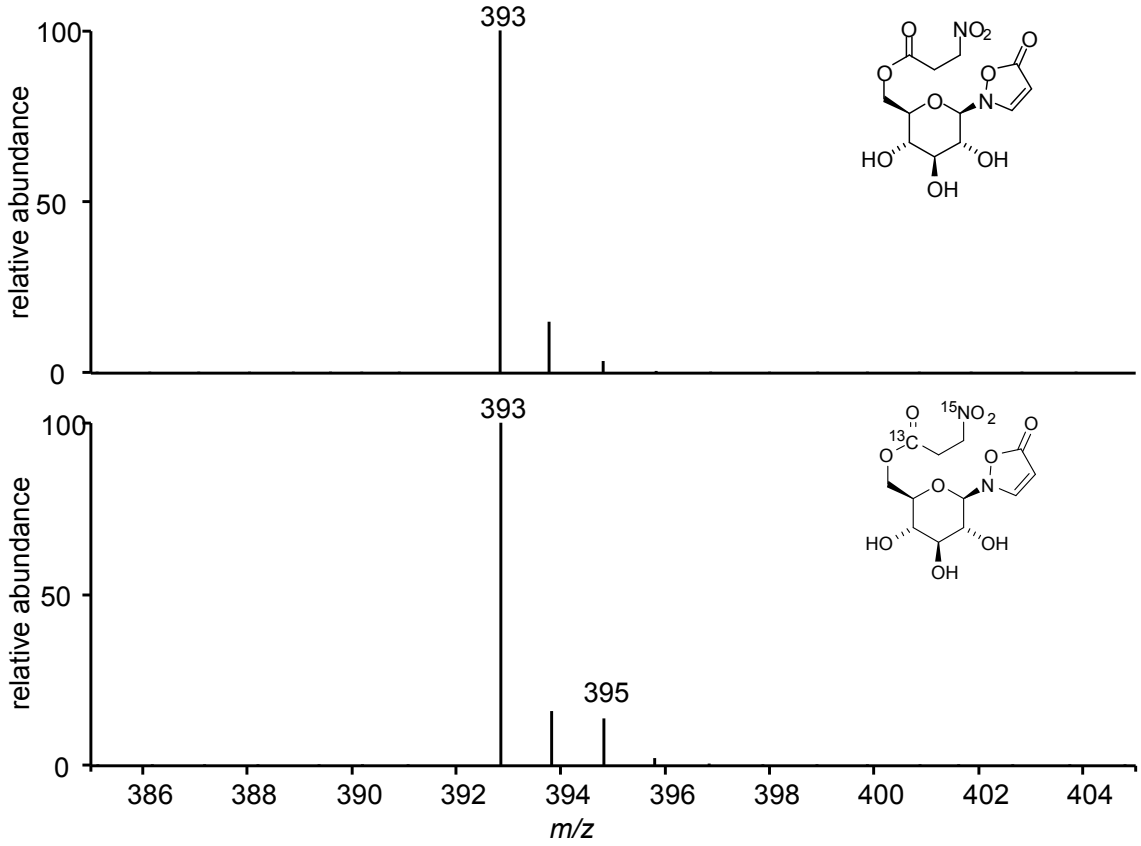

Fig. S2 Mass spectra of isoxazoline-5-one-glucoside ester before and after injection with [^{13}C,^{15}N]-3-NPA.

The signal intensity of *m/z* 395 corresponding to $[M+HCOOH+2]^-$ is increased, see Fig. S5 for labelling ratios of the investigated species

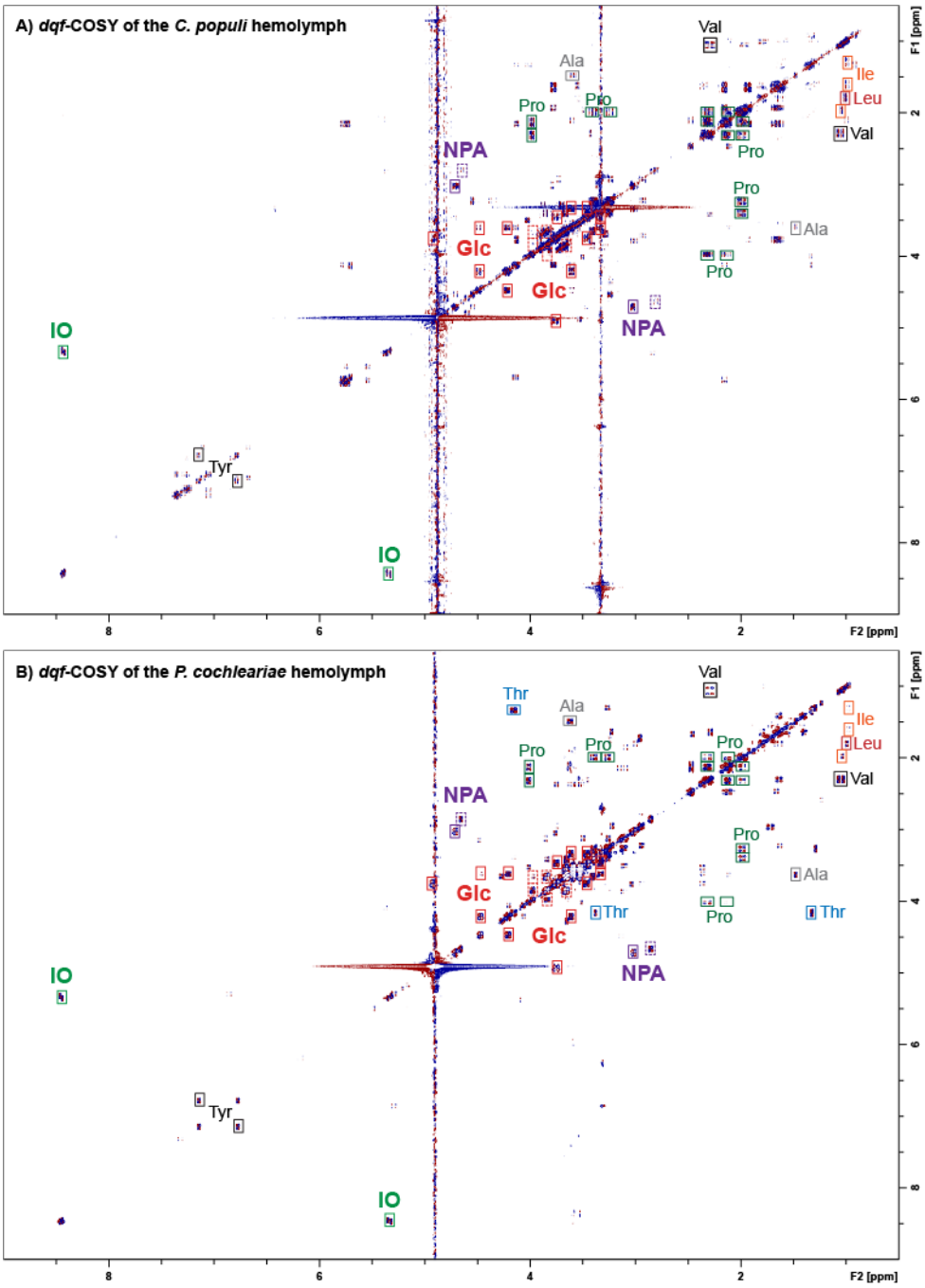

Fig. S3 Double quantum filtered (*dqf*)-COSY spectra (500 MHz, CD_3OD) of the crude hemolymph from *Chrysomela populi* (**A**) or *Phaedon cochleariae* (**B**). Signals corresponding to amino acids (three letter codes), as well as isoxazoline (IO), glucose (Glc), and 3-nitropropionate (NPA) units are labelled.

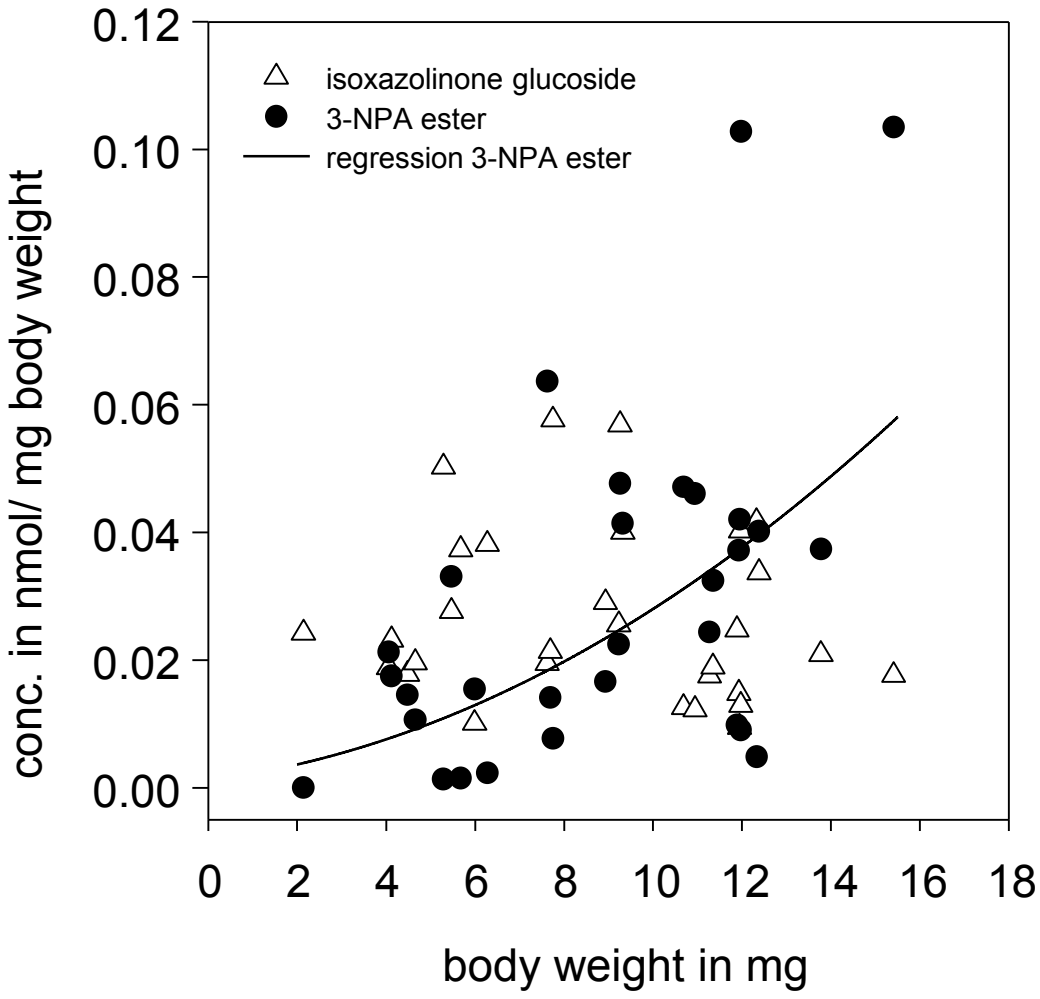

Fig. S4 Concentrations of isoxazolinone glucoside **5** and its 3-Nitropropionic acid ester **6** per mg body weight. The concentration of isoxazolinone glucoside (triangles) remained stable during development (regression analysis, P=0.448, F=0.593, N=30), the ester (dots) showed an increase in concentration (P<0.001, F=14.757, r^2= 0.345, N=30), indicating autogenous synthesis

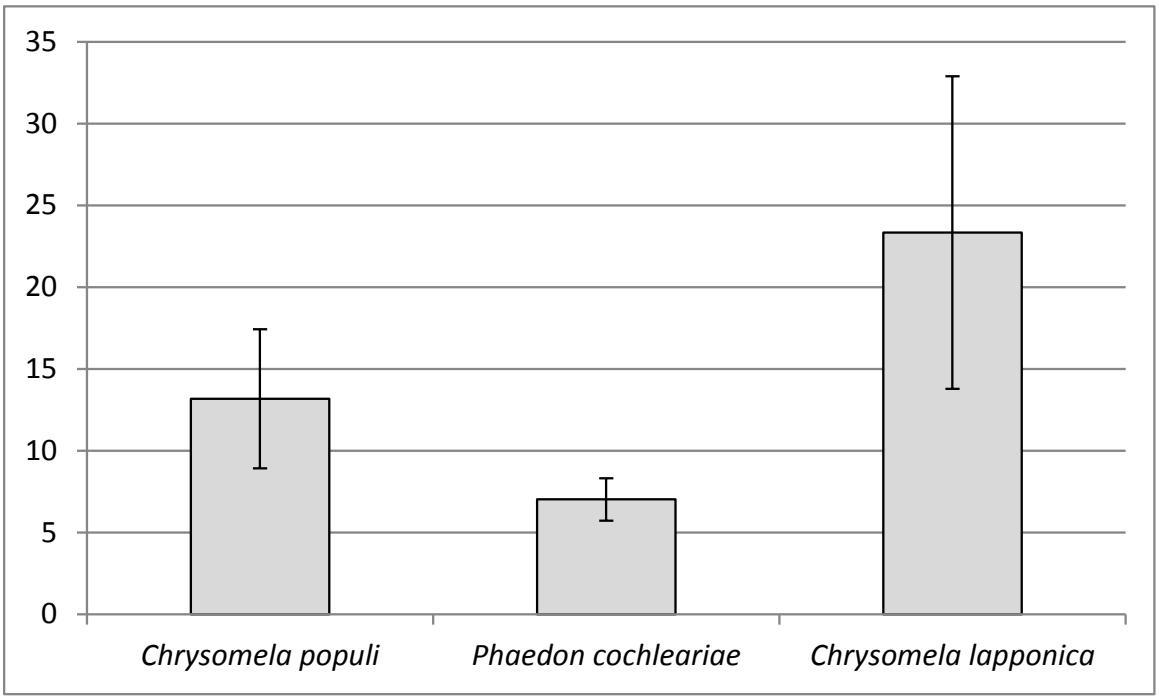

Fig. S5 [^{13}C,^{15}N]-enrichment of isoxazolin glucoside ester **6** upon injection with [^{13}C,^{15}N]-3-Nitropropionic acid. The enrichment of **6** upon incorporation of injected labelled 3-NPA was calculated based on the relative intensities of the isotopic signals (detected as formic acid adducts) by using the equation: [^{13}C,^{15}N]-**6** in % = 100 / ([M+HCOO]$^-$ L + [M+1+HCOO]$^-$ L + [M+2+HCOO]$^-$ L) * ([M+2+HCOO] $^-$ L - [M+2+HCOO] $^-$ C) with L representing the labelled compound and C the unlabelled control. The isotope enrichment for compound **6** in *Chrysomela populi* was 13.2 % ± 4,3% (mean value ± standard deviation, n=10 for each species), in the case of *Phaedon cochleariae* it was 7% ± 1,3%, while *Chrysomela lapponica* showed 24.5% ± 9,5% enrichment.

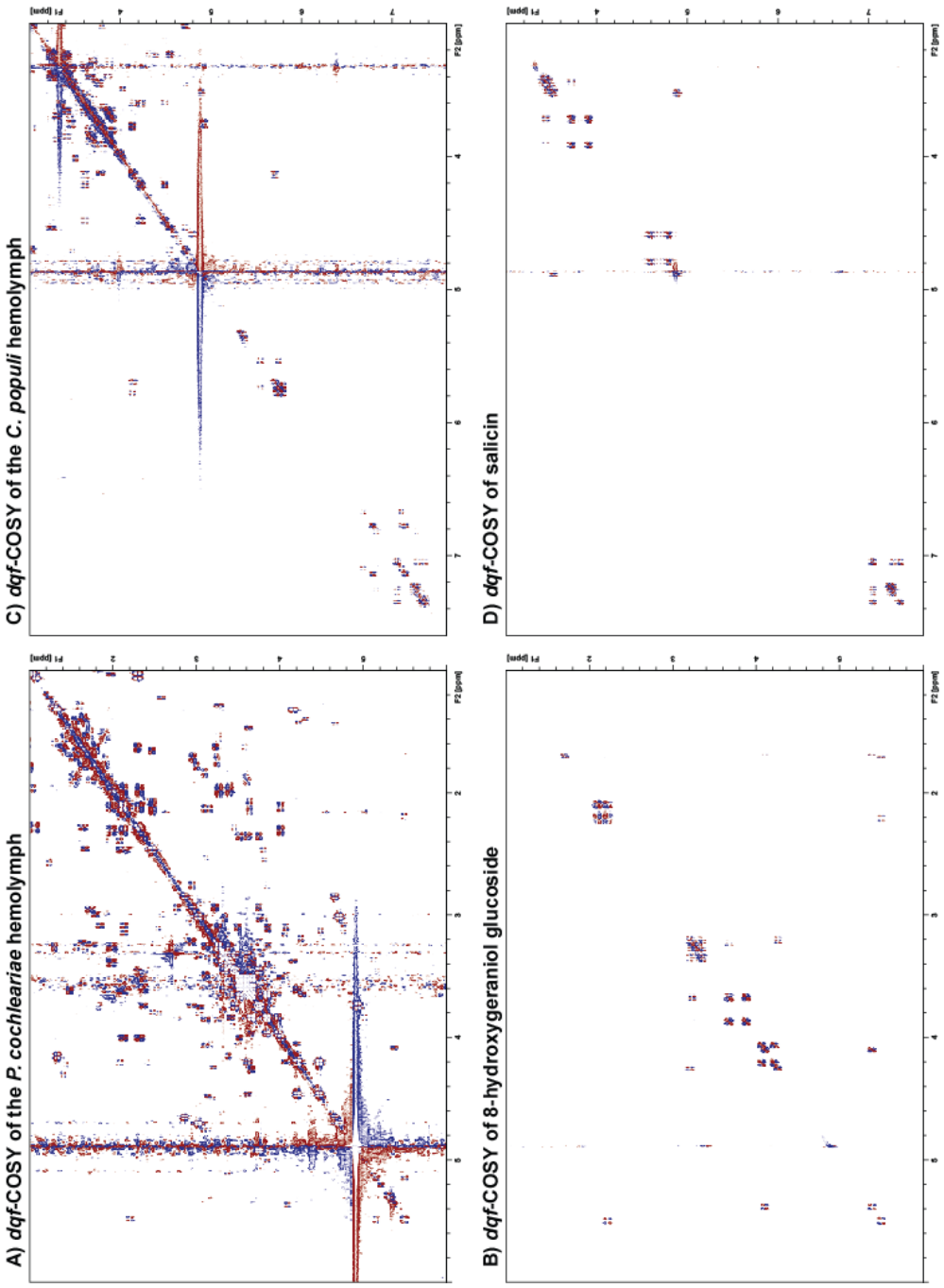

Fig. S6 NMR spectroscopic identification of 8-hydroxygeraniol glucoside and salicin. **A**: Partial *dqf*-COSY spectrum of the crude *Phaedon cochleariae* hemolymph; **B**: *dqf*-COSY spectrum of 8-hydroxygeraniol glucoside standard; **C**: Partial *dqf*-COSY spectrum of the crude *Chrysomela populi* hemolymph; **D**: *dqf*-COSY spectrum of salicin standard.

species	location	plant
Chrysomela lapponica	Uryl, Kazakhstan	*Betula rotundifolia*
Chrysomela 20punctata	Alsemberg, Belgium	*Salix fragilis*
Chrysomela populi	Dornburg, Germany	*Populus trichocarpa*
Chrysomela saliceti	Halle, Belgium	*Salix purpurea*
Chrysomela tremulae	Treignes, Belgium	*Populus tremula*
Linoidea aenea	Treignes, Belgium	*Alnus glutinosa*
Plagiodera versicolora	Alsemberg, Belgium	*Salix fragilis*
Phratora vitellinae	Boitsfort (Brussels), Belgium	*Populus trichocarpa*
Phratora vulgatissima	Buizingen (Halle), Belgium	*Salix cinerea*
Phaedon cochleariae	own breeding	*Brassica rapa* subsp. *pekinensis*
Gastrophysa viridula	Jena, Germany	*Rumex* spp.
Gastrolina depressa	Kyoto, Japan	*Juglans regia*
Chrysolina bruviniensis	Saint-Genesius-Rode, Belgium	*Hypericum perforatum*
Chrysolina coerulans	Brussels, Belgium	*Mentha viridis*
Chrysolina herbaceae	Saint-Genesius-Rode, Belgium	*Mentha viridis*
Leptinotarsa decemlineata	Brussels, Belgium	*Solanum tuberosum*
Agelastica alni	Halle, Belgium	*Alnus glutinosa*

Tab. S1 Leaf beetle larvae analyzed for this study with collection site and host plant

	5				6			
	^{1}H	**COSY**	**^{13}C**	**HMBC**	**^{1}H**	**COSY**	**^{13}C**	**HMBC**
1	4.925 d	$J_{1,2}$ = 9.2	90.4*	C-1', C-2,3,5	4.930 d	$J_{1,2}$ = 9.2	90.2*	C-1' C-2,3,5
2	3.746*	H-1', H-3'	71.0*	C-1,3	3.746*	H-1', H-3'	71.0*	C-1,3
3	3.457*	H-2', H-4'	78.5*	C-2,4	3.457*	H-2', H-4'	78.5*	C-2,4
4	3.326*	H-3', H-5'	70.9	C-3,5,6	3.326*	H-3', H-5'	70.9	C-3,5,6
5	3.407 ddd	$J_{5,4}$ = 10.8 $J_{5,6}$ = 5.5	80.3	C-1,3,4	3.610 ddd	$J_{5,4}$ = 10.7 $J_{5,6}$ = 5.7	77.2	C-1,4
6a	3.84 dd 12.2, 2.1	^{2}J = 12.2 $J_{6,5}$ = 2.1	62.3	C-4,5	4.466 dd 12.0, 1.9	^{2}J = 12.0 $J_{6,5}$ = 1.9	64.7	C-1'', C-4,5
6b	3.65 dd	^{2}J = 12.3 $J_{6,5}$ = 6.0		C-4,5	4.203 dd	^{2}J = 12.0 $J_{6,5}$ = 6.0		C-1'', C-4,5
1'	8.453 d	$J_{1,2}$ = 3.7	154.8	C-2', C-3'	8.433 d	$J_{1,2}$ = 3.7	155.1	C-2', C-3'
2'	5.315 d	$J_{2,1}$ = 3.7	90.9	C-1', C-3'	5.344 d	$J_{2,1}$ = 3.7	91.5	C-1', C-3'
3'	-	-	174.0		-	-	173.9	
1''	-	-	-		-	-	171.5	
2''	-	-	-		3.016 t	J = 5.9	31.7	
3''	-	-	-		4.705 t	J = 5.8	70.7	C-1'', C-2''

Tab. S2 NMR data of isoxazolinone glucosides **5** and **6** derived from analysis of ^{1}H NMR, *dqf*-COSY, HSQC, and HMBC spectra of *Phaedon cochleariae* hemolymph

species	tribe	subtribe	**5**	**6**	3-NPA
Chrysomela lapponica[1] willow	Chrysomelini	Chrysomelina	4,12	3,81	+
Chrysomela lapponica[1] birch	Chrysomelini	Chrysomelina	20,9	1,2	+
Chrysomela 20punctata	Chrysomelini	Chrysomelina	3,81	-	+
Chrysomela populi[1]	Chrysomelini	Chrysomelina	3,13	18,18	+
Chrysomela saliceti	Chrysomelini	Chrysomelina	5,1	1,13	+
Chrysomela tremulae	Chrysomelini	Chrysomelina	1,49	+	+
Linaidea aenea	Chrysomelini	Chrysomelina	11,61	-	+
Plagiodera versicolora	Chrysomelini	Chrysomelina	8,2	-	+
Phratora vittelinae	Chrysomelini	Chrysomelina	9,08	-	+
Phratora vulgatissima	Chrysomelini	Chrysomelina	11,36	-	+
Phaedon cochleariae[1]	Chrysomelini	Chrysomelina	9,05	49,05	+
Gastrophysa viridula[2]	Chrysomelini	Chrysomelina	+	-	+
Gastrolina depressa	Chrysomelini	Chrysomelina	+	-	+
Chrysolina brunsvicensis	Chrysolinini	Doryphorina	-	-	
Chrysolina coerulans	Chrysolinini	Doryphorina	-	-	
Chrysolina herbaceae	Chrysolinini	Doryphorina	-	-	
Leptinotarsa decemlineata	Chrysolinini	Doryphorina	-	-	
Agelastica alni	Sermylini		-	-	

Tab. S3 Screening of larval hemolymph for isoxazolinone glucoside, the corresponding ester and the presence of free 3-Nitropropionic acid. Hemolymph was taken in capillaries, sealed and shipped at RT. [1] hemolymph analyzed freshly. [2] amounts below limit of quantification. Concentrations are given in nmol/mg hemolymph.

8.5. Manuscript 4

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Biosynthesis of isoxazolin-5-one and 3-nitropropanoic acid containing glucosides in juvenile Chrysomelina†

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Stable-isotope-labeled precursors were used to establish the biosynthetic pathway leading from β-alanine towards isoxazolin-5-one glucoside **1** and its 3-nitropropanoate (3-NPA) ester **2** in Chrysomelina larvae. Both structural elements originate from sequestered plant-derived β-alanine or from propanoyl-CoA that is derived from the degradation of some essential amino acids, *e.g.* valine. β-Alanine is converted into 3-NPA and isoxazolinone **5** by consecutive oxidations of the amino group of β-Ala. Substituting the diphospho group of α-UDP-glucose with **5** generates the isoxazolin-5-one glucoside **1**, which serves in the circulating hemolymph of the larva as a platform for esterification with 3-nitropropanoyl-CoA. The pathway was validated with larvae of *Phaedon cochleariae*, *Chrysomela populi* as well as *Gastrophysa viridula*.

Introduction

Leaf beetles of the subtribe Chrysomelina[1–8] and a number of legume plants[9–12] produce the glucosides **1** and **2** (Fig. 1). The latter serves as a pre-toxic storage compound for the actual poison 3-nitropropanoic acid (3-NPA). 3-NPA derived compounds provide a second defensive line in Chrysomelina, parallel with and independent of larval defensive secretions released from nine paired dorsal glands.[13,14]

Free 3-NPA was found in low concentration in the hemolymph of Chrysomelina, but was observed in significant amounts in adult secretions of these beetles as well as in some plants, *e.g. Corynocarpus laevigatus*, and fungi, for example in *Penicillium atrovenetum*.[15–19] The toxic effect of this compound is due to its isoelectronic character to succinic acid, leading to a covalent addition product with mammalian succinate dehydrogenase.[20,21] Thus, mitochondrial respiration is inhibited in these animals. As this effect is most relevant to nerve cells[22] significant economic damage is caused to cattle feeding on 3-NPA containing food plants.[23]

Aspects of the biosynthesis of 3-NPA have been characterized in *Penicillium atrovenetum* using stable-isotope-labelled precursors, *e.g.* [2-^{13}C,^{15}N]-asp and $^{18}O_2$.[17,18,24] In plants, the biosyntheses of isoxazolin-5-one derivatives (glucosides and non-glucosides)[9,25,26] as well as of the 3-NPA moiety[24,27] have been examined using ^{14}C-labeled compounds or with *in vitro* assays. In *Indigofera spicata* 3-NPA derives from malonate and malonyl monohydroxamate.[27] The biosynthesis might involve β-alanine as an intermediate.[28]

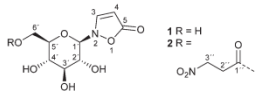

Fig. 1 Isoxazolin-5-one glucosides in Chrysomelina larvae.

Adult leaf beetles of *Chrysomela tremulae* feeding on host plant leaves impregnated with [$^{14}C_4$]-aspartate demonstrated incorporation of radioactivity into compounds **1** and **2**.[8] This experiment indicates the ability for *de novo* production of compounds **1** and **2** in Chrysomelina. Since no evidence was provided for any suggested intermediate, a contiguous metabolic pathway for the biosynthesis of compounds **1** and **2** in Chrysomelina leaf beetles has not yet been elucidated.[6,8]

Results and discussion

Along with previous identifications of 3-NPA in the defensive secretions of adult leaf beetles[6,8] the first biosynthetic experiments with [$^{14}C_4$]-aspartate were published claiming aspartate as the ultimate precursor.[8] As shown in Fig. 2, feeding of [$^{13}C_4$^{15}N]-Asp, surface-impregnated on leaves of *B. rapa pekinensis* and fed to larvae of *Phaedon cochleariae* (see Experimental) results in a cluster of ions in the area of the quasimolecular ion of **2** (formate adduct; m/z = 393 to 401)

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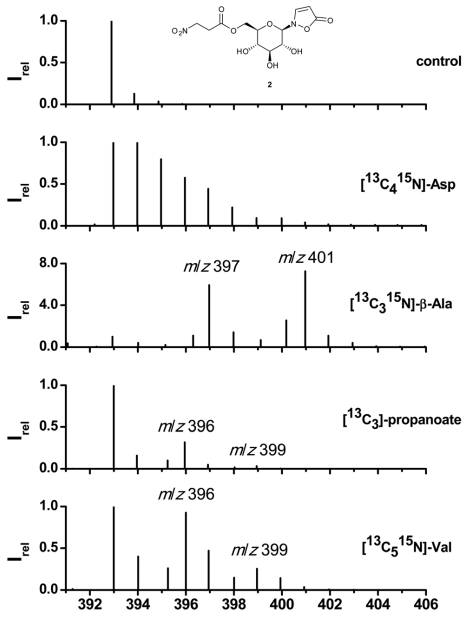

Fig. 2 Representative mass spectra of compound **2** after LC separation of larval extracts (MeCN/H_2O, 1 : 1) from *P. cochleariae* after feeding on different diets for 10 d; diets consisted of *B. rapa pekinensis* leaves, impregnated with KH_2PO_4/K_2HPO_4 buffered solutions of the compounds given above; as a control only blank buffer was used.

which is composed of labeled and randomly re-assembled fragments of [$^{13}C_4{}^{15}N$]-Asp. In contrast, administration of [$^{13}C_3{}^{15}N$]-β-Ala displays a distinct pattern of isotopomers consistent with the incorporation of an intact C_3-segment and the ^{15}N of the fed [$^{13}C_3{}^{15}N$]-β-Ala (*m*/*z* = 397) into **1**. The 3-NPA ester **2** comprises two contiguous units of [$^{13}C_3{}^{15}N$]-β-Ala as is obvious from the fragment at *m*/*z* = 401. Peaks arising at *m*/*z* = 396 or 400 most likely result from loss of the nitrogen atom during transamination or from incomplete labelling of the commercial [$^{13}C_3{}^{15}N$]-β-Ala. Since β-alanine can be sequestered or originate from essential amino acids such as Val, Thr, Met, or Ile,[29–31] *via* propanoate as an intermediate, its origin was further addressed by feeding [$^{13}C_3$]-propanoate and [$^{13}C_5{}^{15}N$]-valine (Table S1†). In both cases the distinct fragment pattern from the incorporation of an intact carbon skeleton of the administered precursors was maintained. In the case of [$^{13}C_3$]-propanoate two fragments at *m*/*z* = 396 and 399 support incorporation of one intact propanoate moiety into **1** and up to two units into **2**. The same pattern is observed after feeding of [$^{13}C_5{}^{15}N$]-valine, while the ^{15}N of the precursor amino acid is lost. This loss of the nitrogen atom together with consecutive incorporation by later transamination can lead to either [M + 4]- and [M + 5]-peaks ([M + 7]- and [M + 8]-peaks respectively), when the nitrogen is incorporated into ^{13}C-labelled precursors, or [M + 1]- and [M + 2]-peaks, if the labelled nitrogen is incorporated into natural unlabeled precursors.

The HR-MS analysis of hydrolytically cleaved free 3-NPA from **2** confirms these findings. Feeding of [$^{13}C_3{}^{15}N$]-β-Ala generates a molecular ion at *m*/*z* = 122.021471 ($^{13}C_3H_4{}^{15}NO_4$, calc. for 122.021681, [M − H]$^-$; Fig. S4†). The carbon skeletons of [$^{13}C_4{}^{15}N$]-Thr, [$^{13}C_3{}^{15}N$]-Ala, and [$^{13}C_2$]-malonate were not incorporated at all into either **1** or **2** (Table S1†).

To explore the probability of the incorporation (%, see Experimental) of plant-derived β-alanine *versus* the *de novo* synthesis of β-Ala from essential precursor amino acids,[29–31] defined amounts of [$^{13}C_4{}^{15}N$]-Asp, [$^{13}C_3$]-propanoate, and [$^{13}C_3{}^{15}N$]-β-Ala were injected into the hemolymph of *P. cochleariae* larvae (see Experimental; Table S1†) and the products **1** and **2** were analyzed by mass spectrometry. In the case of Asp no significant incorporation into **1** or **2** was observed (0.019 ± 0.241%), while average values of 2.3 ± 1.5 and 17.4 ± 11.2% were determined for labelled propanoate and β-Ala, respectively. Altogether, these results indicate that aspartate catabolism plays no significant role in the biosynthesis of compounds **1** and **2** although Asp is abundantly present in food plants (Fig. S13†). Altogether, we conclude that **1** and **2** are produced from both, sequestered and *de novo* produced β-Ala from degradation of the appropriate essential amino acids present in the food plant.

To investigate the later steps of the metabolic route from β-alanine to the isoxazolinone glucoside **1** and to its 3-NPA ester **2**, potential intermediates such as [1-$^{13}C^{15}N$]-**3** and [1-$^{13}C^{15}N$]-**4** were synthesized and injected into the larvae (Table S1†). Accordingly, a stepwise oxygenation at the nitrogen atom of β-alanine first produces (*N*-hydroxyamino)propanoic acid **3** and (*N*-hydroxyimino)propanoic acid **4** (Fig. 3) after elimination of water from the postulated and unstable *N*-dihydroxy precursor. Cyclization of **4** generates the isoxazolinone **5** that is condensed with activated α-UDP-glucose to the isoxazolinone glucoside **1**. Evidence for this reaction was provided by ^1H NMR measurements after incubation of fat body samples of all three investigated species together with the substrates (Fig. S2†). The isoxazolinone glucoside **1** circulates in the hemolymph and serves as a platform for acylation with activated 3-NPA.

Further oxidation of the geminal dihydroxy intermediate of β-Ala generates 3-NPA that is subsequently bound to the C(6′)-hydroxy group of the isoxazolinone glucoside **1**. Activation of 3-NPA is achieved as a CoA-ester and requires ATP (Fig. S3†). The increase of the glucoside ester **2** in later stages of larval development is in line with a decrease of **1** suggesting a tight control of the individual steps of the biosynthesis of **1** (Fig. S6–S11†). A similar trend was observed for **1** and **2** in the hemolymph.[1] Only very small amounts of free 3-NPA were detected in fresh hemolymph samples.[1] Furthermore, neither free β-Ala nor other intermediates, such as **3** to **5** could be detected after silylation and GC-MS analysis of hemolymph samples or whole larval extracts.

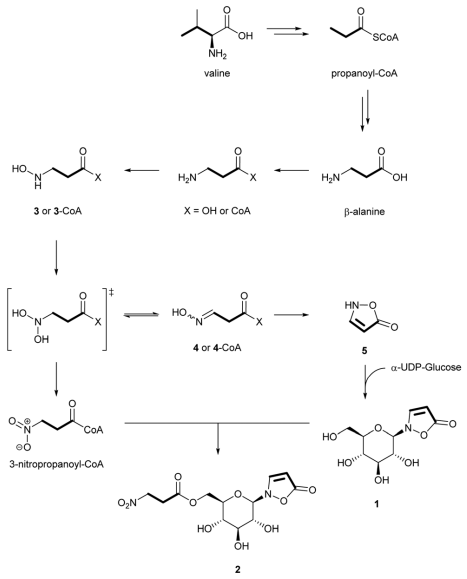

Fig. 3 Proposed biosynthesis of compounds 1 and 2 in Chrysomelina larvae.

Conclusion

Beetles produce 3-NPA- and the isoxazolinone-moiety from plant-derived β-alanine or by degradation of the appropriate amino acids such as L-Val *via* propanoate to β-Ala. The extent of these alternative routes most likely depends on the contents of the required amino acids in the food plants (Fig. S12 and S13†). Since aspartate is not a precursor of **1** or **2**, this excludes a hidden contribution from the gut microbiome since the decarboxylation of aspartate to β-alanine is known from microbial metabolism.[32] It is important to note that the oxidation products of β-alanine serve as precursors for both 3-NPA and the isoxazolinone **1**. As the production of the isoxazolinone glucoside **1** during the very early larval stages precedes the formation of 3-NPA, most likely different oxidases are involved that are tightly regulated. The larval enzymes are still unknown, but show mechanistic similarities to recently described microbial enzymes.[28] The isoxazolinone glucoside **1** circulates in the hemolymph of the insect and serves as a platform for acylation with activated 3-NPA (as CoA ester) as was demonstrated by injection of labelled free 3-NPA into the hemolymph of the larvae, which was then rapidly bound[1] to the glucoside carrier **1**. The steric and electronic features of *N*-glucoside prevent an export of this compound from the hemolymph into neighbouring tissue or the defensive secretion of the insects, although *S*- and *O*-glucosides, for example plant-derived salicin, are sequestered by the larvae and channeled *via* the hemolymph through the whole body into the glandular system for defense production. Both, the *O*- and the *N*-glucosides share the hemolymph as a common carrier medium, but only the natural *O*-glucosides and their synthetic *S*-analogs are allowed to pass the separating membranes.[33] Therefore, the isoxazolinone glucoside **1** has never been found outside the hemolymph of larval systems. This is different in adults, which secrete several 3-NPA esters of **1** on their elytra[15,34] when endangered by a predator. We assume that after ingestion of a larva, the 3-NPA esters of **1** are rapidly hydrolyzed in the gut of the predator leading to intoxication. In a living larva, release of free 3-NPA is cured by activation as a CoA-ester and re-esterification to the C(6′) of isoxazolinone glucoside.[1] The identification and cloning of the enzymes catalyzing the transformation of the amino group of β-alanine to the functionally relevant nitro group are the next and urgent steps to understand the underlying mechanisms and the regulation of defense production in leaf beetle larvae.

Experimental

Insect collection and rearing

The procedures were adapted from already described methods.[1] *Chrysomela populi* was collected near Dornburg, Germany (latitude 51.015, longitude 11.64), on *Populus canadensis*. The beetles were propagated using a light/dark cycle of 16 h light and 8 h darkness (LD 16/8), at 18 ± 2 °C in light and 13 ± 2 °C in darkness. *Gastrophysa viridula* was collected in Jena (latitude 50.929, longitude 11.597). *Phaedon cochleariae* (F.) was reared on *Brassica rapa* subsp. *pekinensis* (*B. rapa pekinensis*) "Cantonner Witkrop" (Quedlinburger Saatgut, Quedlinburg, Germany) and *Gastrophysa viridula* was reared on *Rumex obtusifolius* in a Snijder chamber (Snijders Scientific, Tilburg, Netherlands) in a light/dark cycle of 16 h light and 8 h darkness (LD 16/8) and 13 °C/11 °C ± 1 °C.

Insect sample preparation

150 µl of MeCN/H_2O 1 : 1 were added to individual larvae in grinding tubes equipped with 2 steel beads (diameter/bead = 4.5 mm). The samples were ground with a geno grinder (1210 rpm, 1 min) at rt for each larva. Then the mixtures were centrifuged at 40 °C and 13 000 rcf for 30 min. 110 µl of the supernatant was transferred to a second tube. The samples were stored at −25 °C and vortexed for 15 s directly before analysis by HPLC/MS. All *in vivo* measurements are single-larva analyses. All replicates are biological replicates of individual larvae.

Insect dissection

The larvae were immersed in liquid nitrogen for 30 s. Then the fat body was isolated after cutting the cuticles of the larvae with micro scissors. The tissue was stored at −80 °C prior to use.

Statistical evaluation

The results of the treated larvae were compared to the results of larvae of the control groups, in order to determine the statistical significance of the difference of the mean values. First, the data were analyzed by the Shapiro–Wilk normality test. For normally distributed datasets, the *t*-test was applied. For differently distributed datasets, the Mann–Whitney rank sum test was applied. Statistical difference is defined by a confidence level of at least 95% (* ≙95% ≙0.05, ** ≙0.01, *** ≙0.001).

HPLC/MS analysis

The analysis of compounds **1** and **2** was done by modifying the procedure that appears in the literature.[1] Measurements were carried out on an Agilent HP1100 HPLC system equipped with an OH-endcapped RP-C18 column (RP-C18e), LiChroCART (250 × 4 mm, 5 μm; Merck KGaA, 64271, Darmstadt, Germany) connected to a Finnigan LTQ (Thermo Electron Corp., Dreieich, Germany) ion trap mass spectrometer operating in the APCI mode (vaporizer temperature: 500 °C, capillary temperature 300 °C). Standard compounds for identification were either purchased (Sigma-Aldrich (St Louis, MO, USA)) or synthesized. 2 to 5 μl of the sample volume was injected, depending on the larval size (up to 20 mg larval fresh weight: 5 μl; $m >$ 20 mg: 2 μl). The following parameters were used: flow rate = 0.5 ml min^{-1} at rt: 90% solvent A (H_2O + 0.1% v/v HCO_2H) and 10% solvent B (MeCN + 0.1% v/v HCO_2H) for 5 minutes, linear gradient to 100% solvent B in 5 min, then 100% B for 2 min, linear gradient to 10% B in 5 min and further elution with 10% B for 5 min. For identification and quantification, the signals of the formic acid adducts $[M + HCO_2H - H]^-$ were analyzed (*m/z* 292 for 2-(β-D-glucopyranosyl)-3-isoxazolin-5-one (**1**) and *m/z* 393 for 2-[6′-(3″-nitropropanoyl)-β-D-glucopyranosyl]-3-isoxazolin-5-one (**2**)). The column was washed for 20 h at 40 °C with 2-propanol/MeCN 1 : 1 and then equilibrated to H_2O/MeCN 9 : 1 prior to use.

GC/MS analysis

The measurement parameters were similar to the literature protocols.[35] 1 μl of each sample was injected at a split ratio of 1 : 25 into a GC/MS system equipped with an A 200S autosampler, a GC 2000 gas chromatograph, and a Voyager quadrupole mass spectrometer including a dynode/phosphor/photomultiplier detector (all ThermoQuest, Manchester, UK). Tris(perfluorobutyl)amine (CF43) was used as a reference gas for tuning. Mass spectra were recorded from *m/z* 50 to 622 at 0.53 s $scan^{-1}$ for trimethylsilylated samples (TMS). For quantifications, the parameters were as follows: an injection temperature of 230 °C was chosen, the interface temperature was adjusted to 250 °C, and the ion source temperature was 200 °C. Helium flow was 1.5 mL min^{-1}. After a 5 min solvent delay at 70 °C, the oven temperature was increased by 5 °C min^{-1} to 140 °C, then by 40 °C min^{-1} to 310 °C; the temperature was constant for 1 min, then cooled to 70 °C and equilibrated for 5 min. Ion trace integration was performed manually over the complete intensities of the signals.

NMR and HRMS analysis

NMR spectra were recorded using a Bruker-Spektrospin AVANCE 400 UltraShield spectrometer operating at 400 MHz (^1H) and 100 MHz (^{13}C). Chemical shifts (δ) are quoted in parts per million (ppm) and are referenced to the signals of residual protonated solvents in ^1H spectra ($CHCl_3$ at δ 7.26 ppm; HDO at δ 4.79 ppm) and deuterated chloroform in ^{13}C spectra ($CDCl_3$ at δ 77.16 ppm). Acetonitrile was added as a reference for ^{13}C NMR spectra in D_2O (H_3CCN at δ 1.47 ppm).[36] The multiplicities are given as follows: s, singlet; d, doublet; t, triplet, dd, doublet of doublets; dt, doublet of triplets; q, quartet; dq, doublet of quartets; m, multiplet. High-resolution mass spectra were recorded on a Bruker Maxis UHR-qTOF mass spectrometer.

Syntheses

Modified literature protocols were used to synthesize [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**,[37] [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**,[38] 2,2,2-trichloroethyl-3-nitropropanoate (**6**),[38] isoxazolin-5-(2*H*)-one (**5**),[39] [1-$^{13}C^{15}N$]-3-(hydroxyamino)propanoate (**3**)[18,40] and [1-$^{13}C^{15}N$]-3-(hydroxyimino)propanoate (**4**).[39] The details of the synthetic protocols as well as the analytical data are presented in the ESI (S5–S16).†

In vitro assays

To show the incorporation of isoxazolin-5-one **5** into compound **1**, 10 mg of the isolated fat body tissue was suspended in 400 μl H_2O. In addition, one solution containing 2.6 mg of isoxazolin-5-one **5** and one solution containing 18.7 mg of commercial α-UDP-glucose each in 200 μl were prepared. Solutions of compound **5** and α-UDP-Glc were mixed and split again into two solutions of 200 μl. To one solution, 200 μl of the fat body suspension was added; to the residual solution, 200 μl of buffer (KH_2PO_4/K_2HPO_4, 100 mM) was added. After 1 d of incubation at 30 to 40 °C, 400 μl of D_2O was added to each solution, including the fat body suspension, after which the mixtures were centrifuged and analyzed by 1D ^1H NMR experiments (512 scans each).

The ATP/CoA-dependent incorporation of 3-NPA into compound **2** was shown according to procedures in the literature[41] Solutions of ATP (c = 125 mM), CoA (c = 5 mM), 3-NPA (10.5 mM), compound **1** (10.5 mM) and the isolated fat body tissue (m = 30 mg) in 2 ml of buffer (Tris-base 50 mM, sucrose 250 mM, $MgCl_2$ 2 mM, 1 μl ml^{-1} dithiothreitol solution, 10 μl ml^{-1} protease inhibitor mix) were prepared. The fat body mixture was homogenized using a geno grinder (1200 rpm, 30 s). Then five mixtures with an individual volume of 1 ml were prepared from these solutions as follows: solution 1 containing ATP, CoA, 3-NPA, compound **1**, fat body; solution 2 (CoA, 3-NPA, compound **1**, fat body, buffer); solution 3 (ATP, CoA, 3-NPA, compound **1**, buffer); solution 4 (ATP, 3-NPA, com-

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pound **1**, fat body, buffer) and solution 5 (ATP, CoA, fat body, 2 × buffer). After 1 d of incubation at 30 °C, 200 µl was taken and centrifuged (13 000 rcf, 15 min), and the supernatant was directly analyzed by HPLC/MS.

In vivo injection experiments

The larvae were fixed with pincers manually upon ice under a light microscope. Then a dose of 20 to 40 nmol substance per mg larval fresh weight was injected with a thin glass capillary attached to a microinjector. Typically injection volumes of 200 nl containing solutions of compounds with a concentration of 0.5 mol l^{-1} dissolved in potassium phosphate (KH_2PO_4/K_2HPO_4) or potassium carbonate (K_2CO_3) buffers were applied. After the total volume was injected, the glass capillary was not removed from each larva for 1 min to prevent direct bleeding. Then the larvae were kept in plastic beakers covered with cardboard with a piece of their host plant leaf (100 to 400 mg) to enable air exchange at rt unless noted otherwise. The injected larvae were incubated for 24 h before being extracted with MeCN/H_2O 1 : 1.

Feeding experiments

Whole leaves were impregnated with aqueous buffered (KH_2PO_4/K_2HPO_4, pH 7.4, 500 mmol l^{-1} + 300 µl of acetone, for reduction of surface tension) solutions of the isotopic-labeled compounds using a brush and given to *P. cochleariae* (L1 and L2) as food. The larvae were extracted after 7 to 10 d of feeding. Freshly impregnated leaves were added two or three times. The concentrations of the isotope-labeled compounds were as follows: [$^{13}C_4{}^{15}N$]-aspartate (c = 100 mmol l^{-1}), [^{15}N]-aspartate (c = 100 mmol l^{-1}), [4-^{13}C]-aspartate (c = 100 mmol l^{-1}), [$^{13}C_4$]-aspartate (c = 100 mmol l^{-1}), [1,3-$^{13}C_2$]-malonate, [$^{13}C_3{}^{15}N$]-α-L-alanine (c = 100 mmol l^{-1}), [^{15}N]-α-L-alanine (200 mmol l^{-1}), [$^{13}C_3{}^{15}N$]-β-alanine (c = 100 mmol l^{-1}), [$^{13}C_3$]-propanoic acid (c = 100 mmol l^{-1}), [$^{13}C_5{}^{15}N$]-valine (200 mmol l^{-1}) and [$^{13}C_4{}^{15}N$]-threonine (200 mmol l^{-1}). An overview is presented in the ESI (Table S1†).

Quantification and identification of amino acids in *B. rapa pekinensis*

The samples were analyzed according to procedures modified from the literature. 200–1000 mg FW of *B. rapa* subsp. *pekinensis* leaves were cut, transferred into a 2 mL tube, and mixed with 100 µl of [$^{13}C_3$, ^{15}N]β-alanine (c = 0.6 mmol l^{-1}). The samples were ground by using a geno grinder (1200 rpm, 3 × 5 min) and shaken at 40–60 °C for 3 h. 1 mL of methanol was added, and the samples were centrifuged for 15 min at 13 000 rcf. The supernatant was pipetted into a screw-top glass tube, and 2.5 ml of MeCN was added. The tubes were equipped with a needle, and the solvents were removed in a desiccator under reduced pressure at rt. This procedure was repeated until the samples were completely dried. To each sample, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was added, and the samples were shaken at 40 °C for 30 min. The clear solutions were directly analyzed by GC/MS. The peak areas of the heavy isotope signals corresponding to the standards and the areas of the normal isotopes were integrated. The ratio of the peak areas gave the molar content of the amino acid $c_{m,AA,leaf}$ in nmol (mg fresh weight)$^{-1}$ according to eqn (1):

$$c_{m,AA,leaf} = \frac{n_{AA}}{m_{leaf}} = \frac{I_{AA} \cdot c_{standard} \cdot V_{standard}}{I_{standard} \cdot m_{leaf}} \quad (1)$$

m_{leaf} = fresh weight of the leaf in mg, n_{AA} = amount of amino acid in nmol, I_{AA} = area of the non-isotopic peak ([M + 0]) of the amino acid, $c_{standard}$ = molar concentration of the added solution of the isotopically labeled amino acid standard in µmol µl^{-1}, $V_{standard}$ = volume of added solution of the isotopically labeled amino acid standard in µl, and $I_{standard}$ = area of the isotopic peak of the isotopically labeled amino acid standard.

Quantification of compounds 1 and 2

Solutions of synthetic [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** (c = 17.33 mmol l^{-1}, V = 10 µl) as well as [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** (c = 14.65 mmol l^{-1}, V = 10 µl) in MeCN/H_2O 1 : 1 were added to each larval extract (SIL-IS[42,43]). The peak areas of the heavy isotope signals in the chromatogram (m/z 298 for compound **1**, m/z 399 for compound **2**) were compared to the peak areas of the normal isotopes (m/z 292 for compound **1**, m/z 393 for comp. **2**). The molar contents $c_{m,analyte,larva}$ of compounds **1** and **2** in nmol (mg larval fresh weight)$^{-1}$ were calculated using eqn (2):

$$c_{m,analyte,larva} = \frac{n_{analyte}}{m_{larva}} = \frac{I_{analyte} \cdot c_{standard} \cdot V_{standard}}{I_{standard} \cdot m_{larva}} \quad (2)$$

m_{larva} = fresh body weight of the extracted larva in mg, $n_{analyte}$ = amount of compound **1** or **2** in nmol, $I_{analyte}$ = area of the non-isotopic peak ([M + 0]) of compound **1** or **2**, $c_{standard}$ = molar concentration of added solution of synthetic comp. [1′,2′,3′,4′,5′,6′-$^{13}C_6$]-**1** or [1′,2′,3′,4′,5′,6′-$^{13}C_6$]-**2** in µmol µl^{-1}, $V_{standard}$ = volume of added solution of synthetic [1′,2′,3′,**4**′,5′,6′-$^{13}C_6$]-**1** or [1′,2′,3′,**4**′,5′,6′-$^{13}C_6$]-**2** in µl, and $I_{standard}$ = area of the isotopic peak ([M + 6]) of comp. [1′,2′,3′,**4**′,5′,6′-$^{13}C_6$]-**1** or [1′,2′,3′,**4**′,5′,6′-$^{13}C_6$]-**2**. The resulting values are presented in the ESI (ESI, Fig. S6–S11†). Compounds containing more than one 3-NPA moiety bound to the sugar residue of an isoxazolin-5-one glucoside were detected in secretions of adult leaf beetles.[15] However, these components were not detected in whole Chrysomelina larvae extracts; thus it is clear that compound **2** is the only 3-NPA based pre-toxic compound found at the larval stage. Consequently, quantification of compounds **1** and **2** provides information about the total amount of isoxazolin-5-one and 3-NPA derivatives in Chrysomelina larvae.

Incorporation of injected compounds

To demonstrate the incorporation of the injected compounds had taken place, the areas of the isotopic peaks of compounds **1** and **2** were divided by the areas of the non-isotopic peaks. These ratios were compared to the ratios determined in the control

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groups. In order to determine the percentile C of the incorporation of the injected compounds in % eqn (3) was used:

$$C = \frac{n_{\mathrm{det}}}{n_{\mathrm{inj}}} \times 100 = \frac{\left(\left(\frac{I_1}{I_{\mathrm{M1}}}\right) \cdot c_{\mathrm{m,compound\,1,larva}} + \left(\frac{I_2 + 2 \cdot I_3}{I_{\mathrm{M2}}}\right) \cdot c_{\mathrm{m,compound\,2,larva}}\right) \cdot m_{\mathrm{larva,end}}}{c_{\mathrm{inj}} \cdot V_{\mathrm{inj}}} \times 100 \quad (3)$$

n_{det} = sum of amounts of detected labeled isoxazolin-5-one and 3-NPA moieties in compound **1** and **2** in nmol, n_{inj} = amount of injected labeled compound in nmol, I_1 peak area of labeled compound **1** (intensity of control peak is always subtracted; if 1 of n labeled atoms is incorporated, the value of I is divided by n; if 2 of n are incorporated I is divided by n and multiplied by 2 and so forth), I_{M1} = area of the non-isotopic peak of compound **1** ($[M_{\mathrm{comp.1}} + 0]$), I_2 = area of the single labeled isotopic peak of comp. **2**, I_{M2} = area of the non-isotopic peak of comp. **2** ($[M_{\mathrm{comp.2}} + 0]$), I_3 = area of the double labeled isotopic peak of comp. **2**, $m_{\mathrm{larva,end}}$ = fresh body weight of the larva after incubation, c_{inj} = molar concentration of the injected compound in nmol nl^{-1}, and V_{inj} = volume of the injected compound in nl. Thus, naturally occurring compounds **1** and **2** were used as internal standards within the same measurement. The ratios of isotopic *versus* non-isotopic peaks determined in the control groups were subtracted from the ratios determined in the treated groups.

Author contributions

T. B. and W. B. planned and designed the project and experiments. T. B. synthesized the compounds. T. B. performed injection and feeding experiments. T. B. carried out the *in vitro* experiments. T. B. prepared the samples. T. B. measured and analyzed all NMR spectra. T. B. and K. P. performed HPLC/MS measurements. T. B. performed GC-MS measurements. T. B. analyzed GC- and LC-mass spectra. T. B. analyzed the statistical data. T. B. and W. B. wrote the manuscript.

Conflict of interest

The authors declare no competing financial interest.

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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

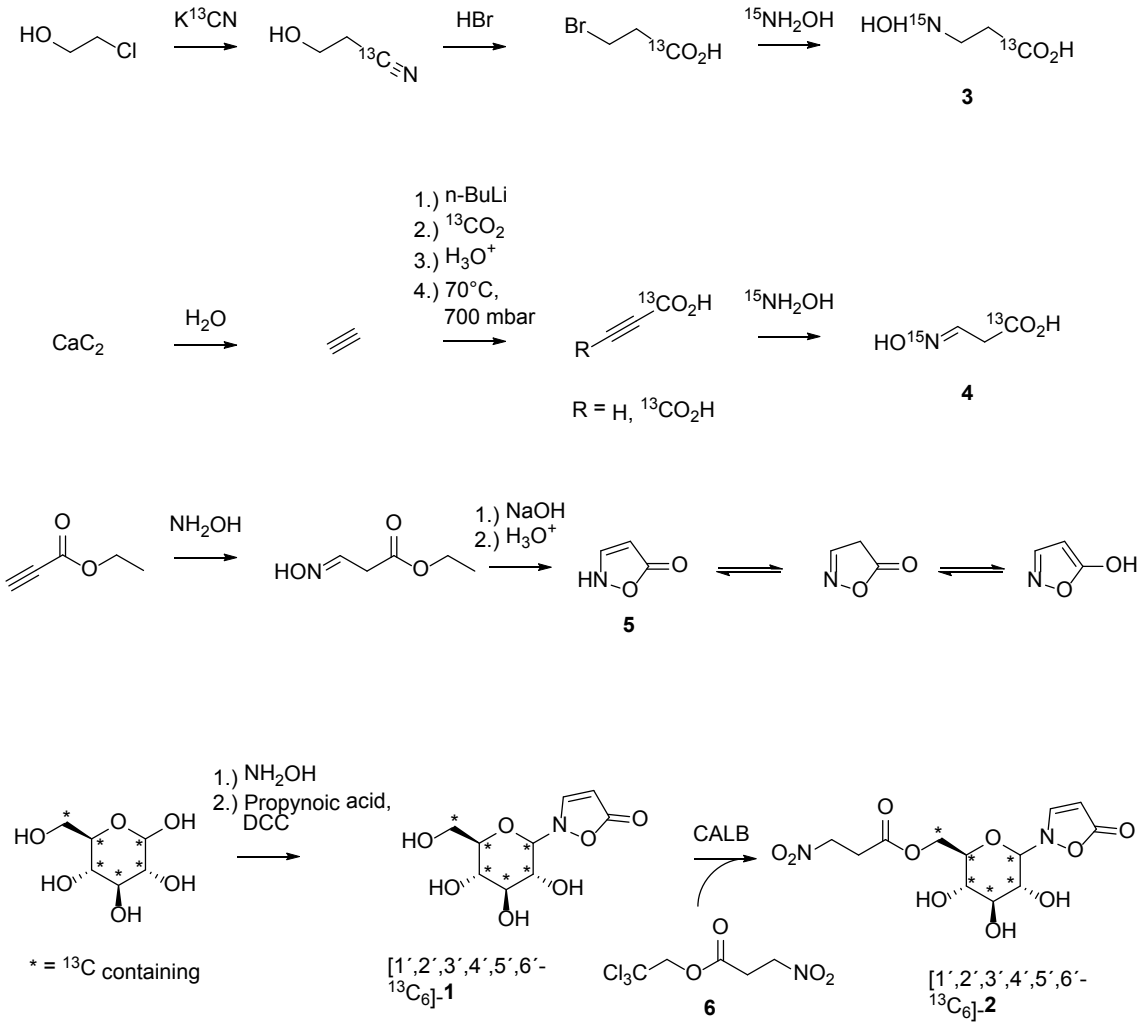


Fig. S1: Synthetic routes for the described substrates **1** to **5**.

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**

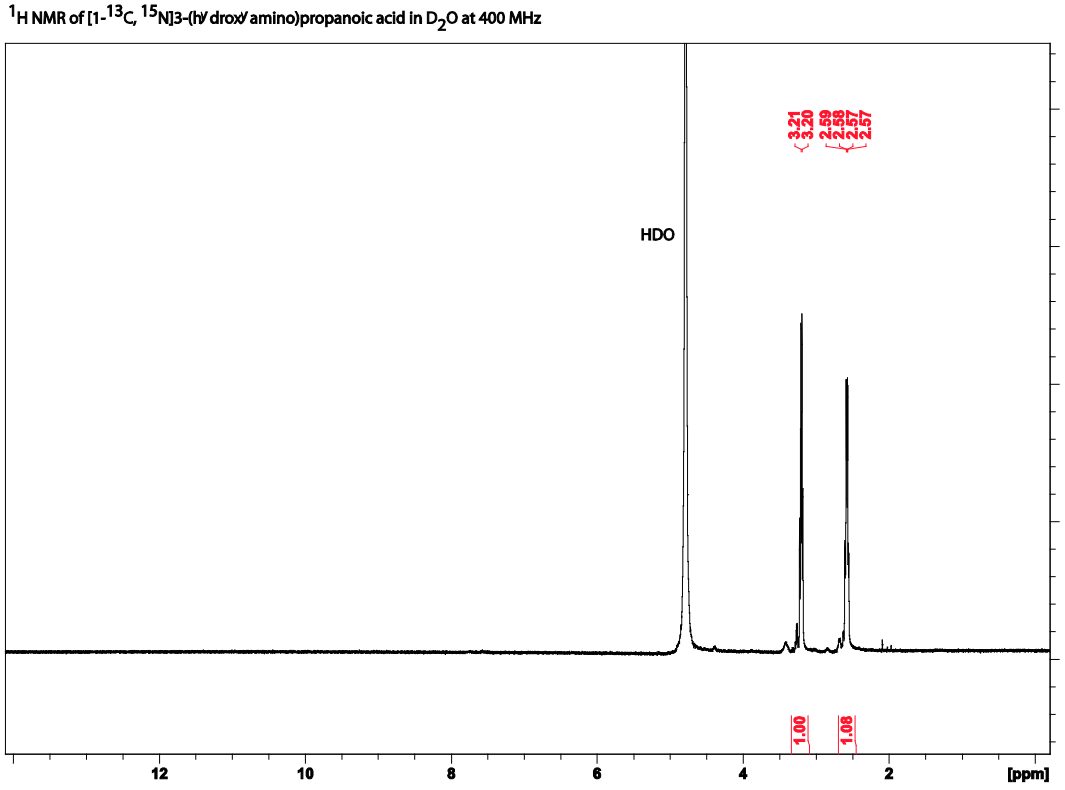
2.223 g (27.61 mmol) of 2-chloroethanol were dissolved in 13.1 ml ethanol and 6.5 ml water. Then 1.034 g (6.9 mmol) NaI and 661 mg (10 mmol) K^{13}CN were added. The mixture was heated to 70 °C for 18h. After the reaction time was finished the solvents were removed at 40 °C under reduced pressure and 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;

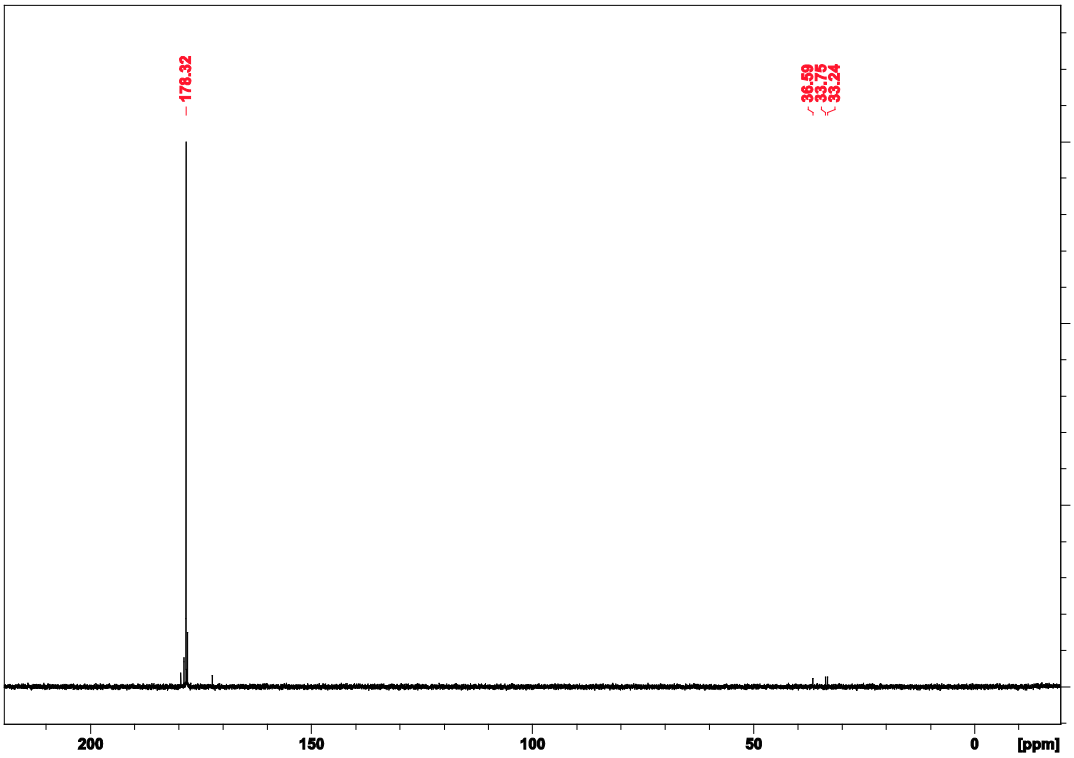
^{1}H NMR (400 MHz, $CDCl_3$) δ 3.21 (q, J = 5.2 Hz, 2H, H-3), 2.58 (dq, J_1 = 6.5 Hz, J_2 = 3.0 Hz, 2H, H-2);

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

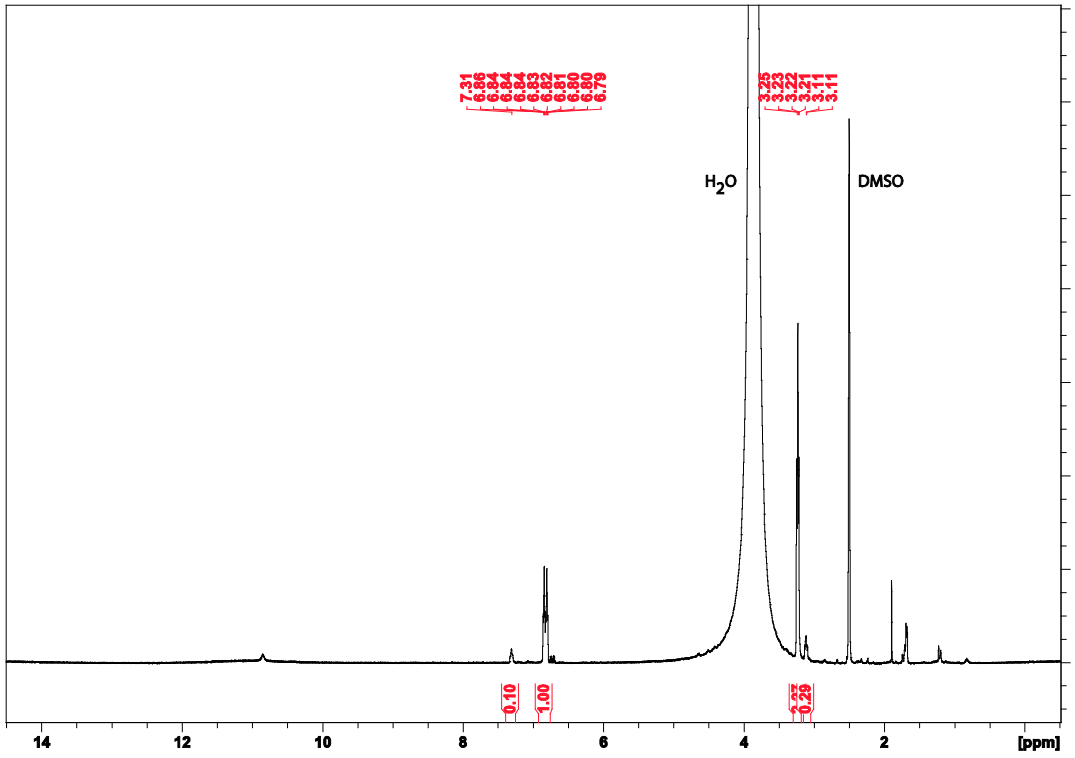
R_f(EtOAc/MeOH/AcOH 100:10:1)=0.63;

^{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);

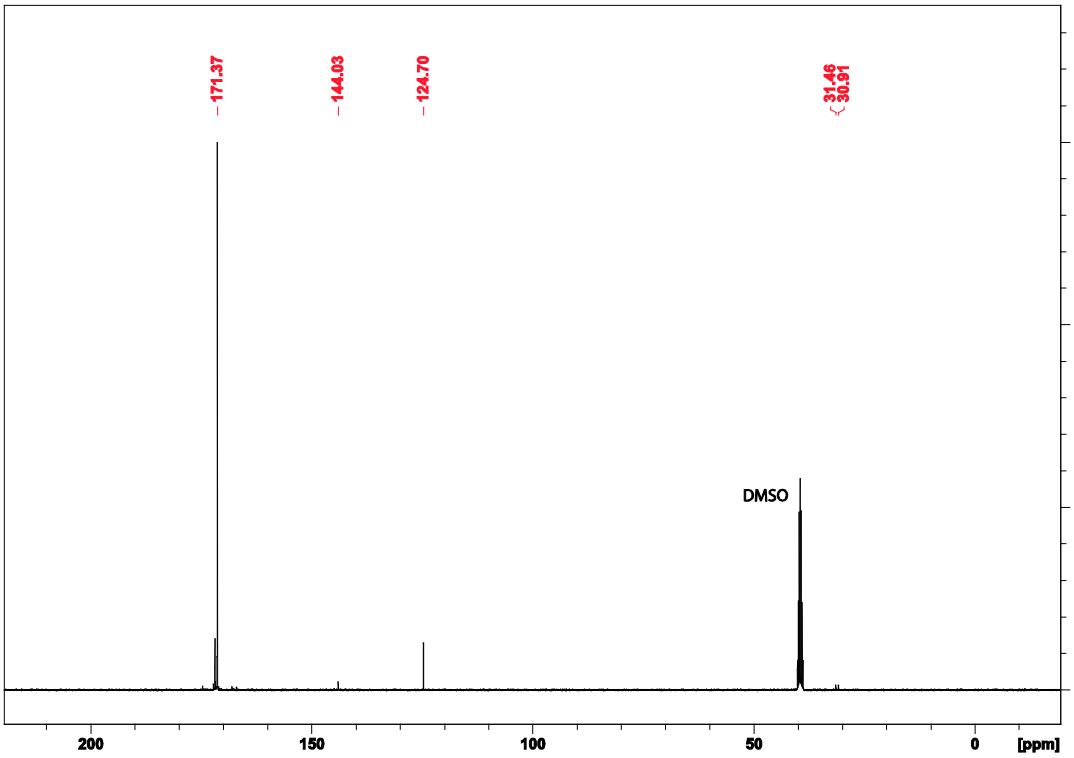
^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4 (s, C-1), 144.0 (s, *Z*-C-3), 124.7 (s, *E*-C-3), 31.2 (d, $^1J_{1,2}$ = 55.7 Hz, C-2);

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**

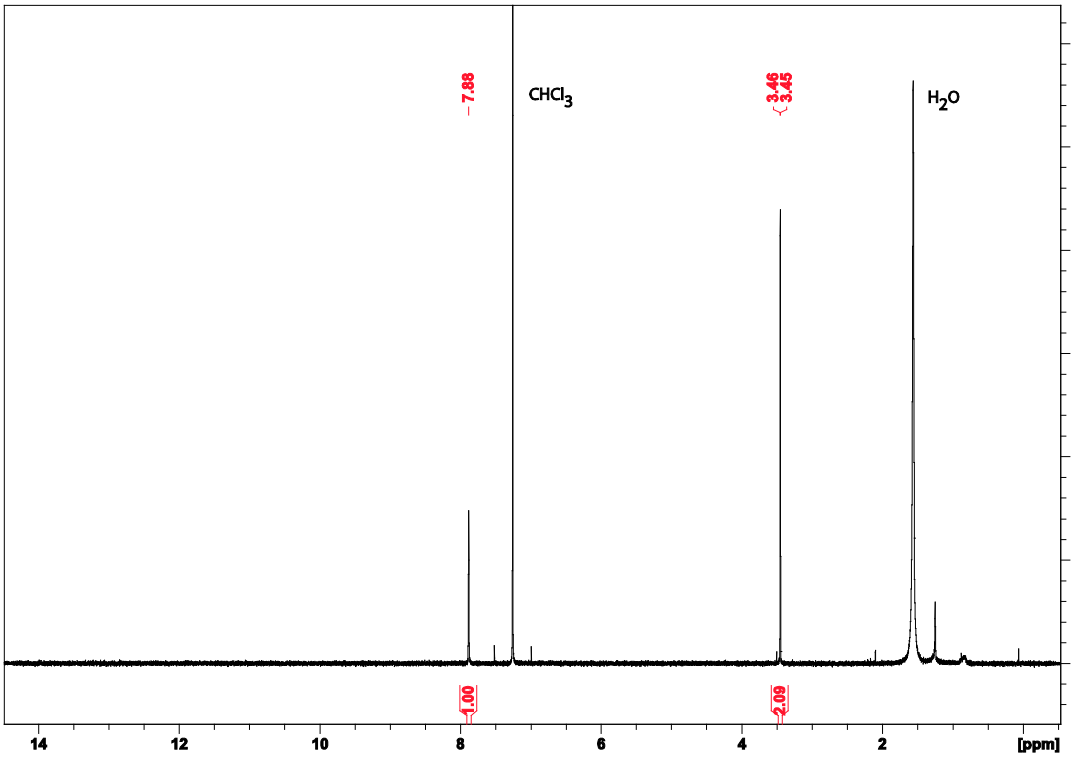
6.57 g (94.6 mmol) NH_2OH*HCl were added at 0 °C to a solution of 21.1 ml KOH in water (c = 4.49 mol/l). Then 4.64 g (47.3 mmol) ethyl propiolate in 37.8 ml ethanol and 9.47 g (94.46 mmol) $KHCO_3$ were added to the mixture at 0 °C. After stirring for 20h at -15 to 7 °C 100 ml of water were added. The 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);

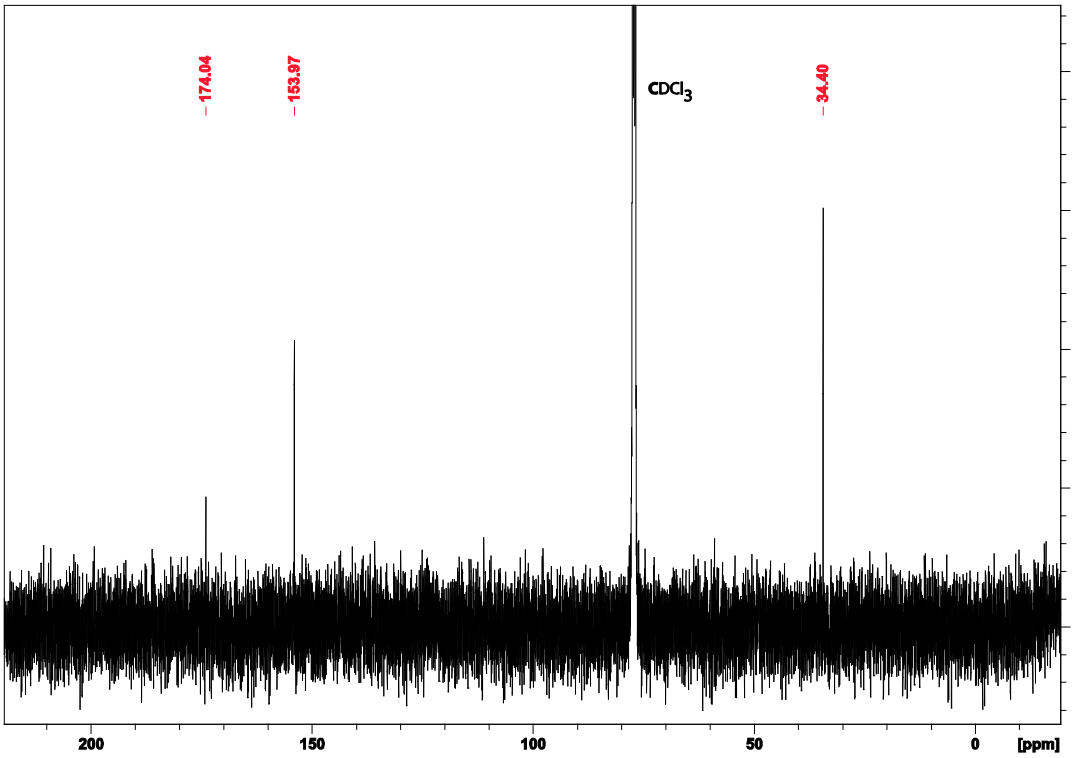
^{13}C NMR (100 MHz, $CDCl_3$) δ 174.0 (C-5), 154.0 (C-3), 34.4 (C-4);

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

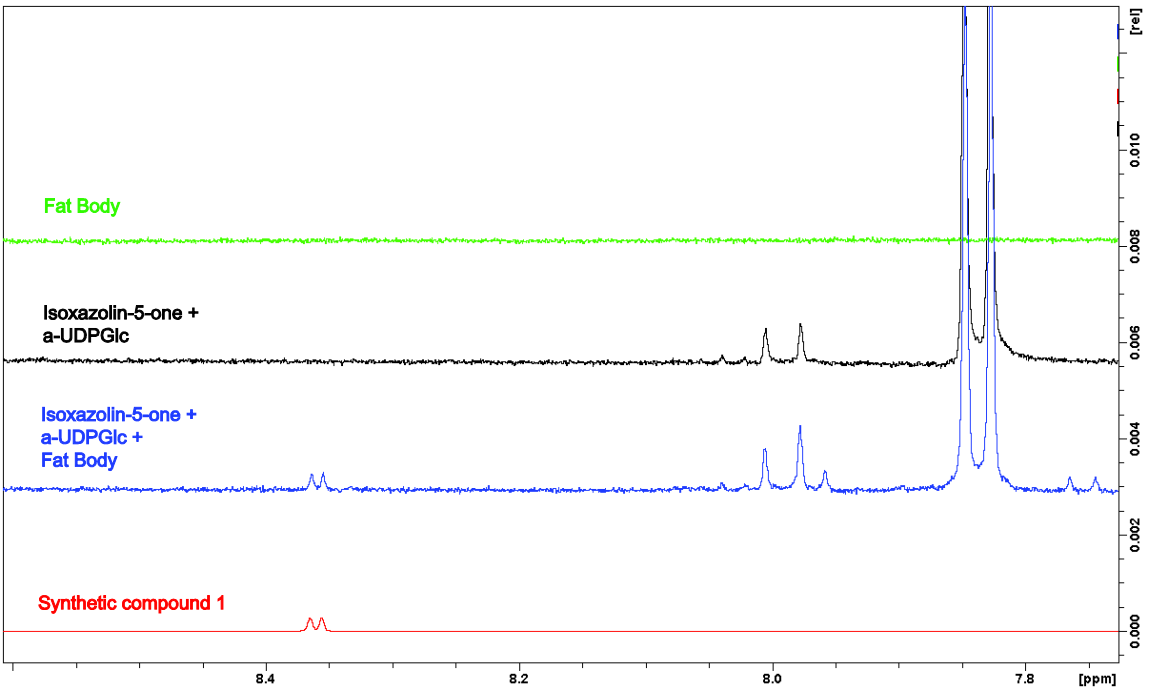


Fig. S2: Representative 400 MHz ^{1}H NMR spectra of buffered solutions of the fat body, as a control, (green) of *Phaedon cochleariae*, compound **5** and α-UDP-Glucose (black), compound **5**, α-UDP-Glucose and fat body (blue) as well as the synthetic compound **1** (red) after 1d of incubation at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

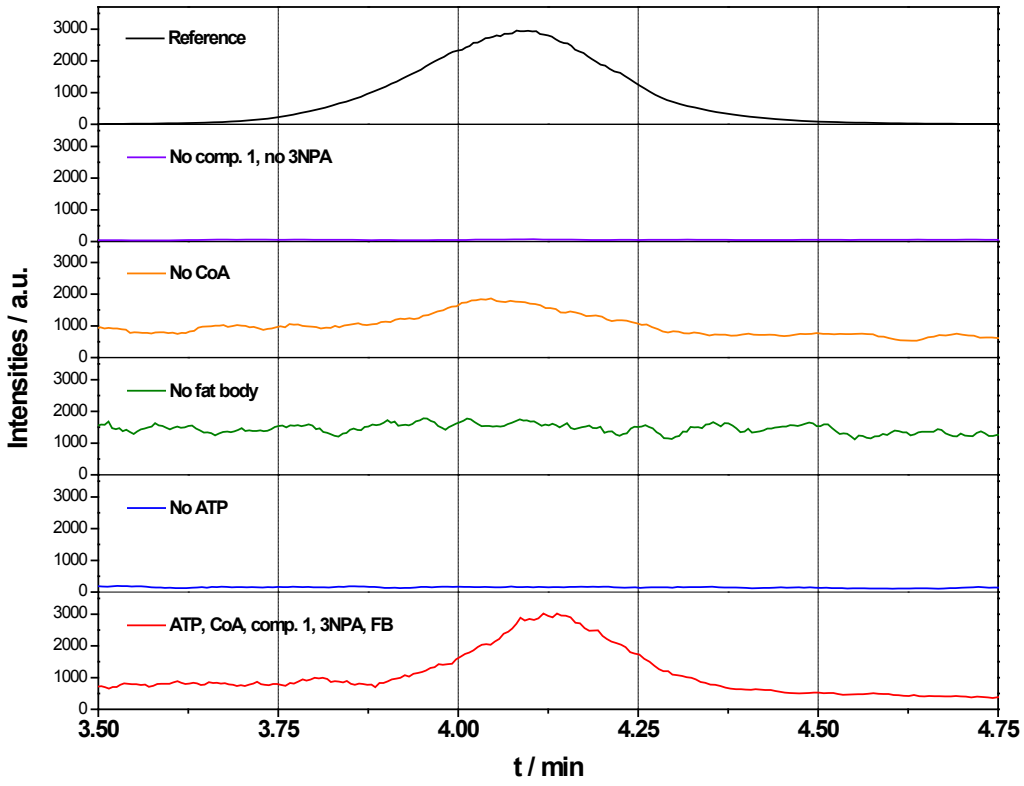
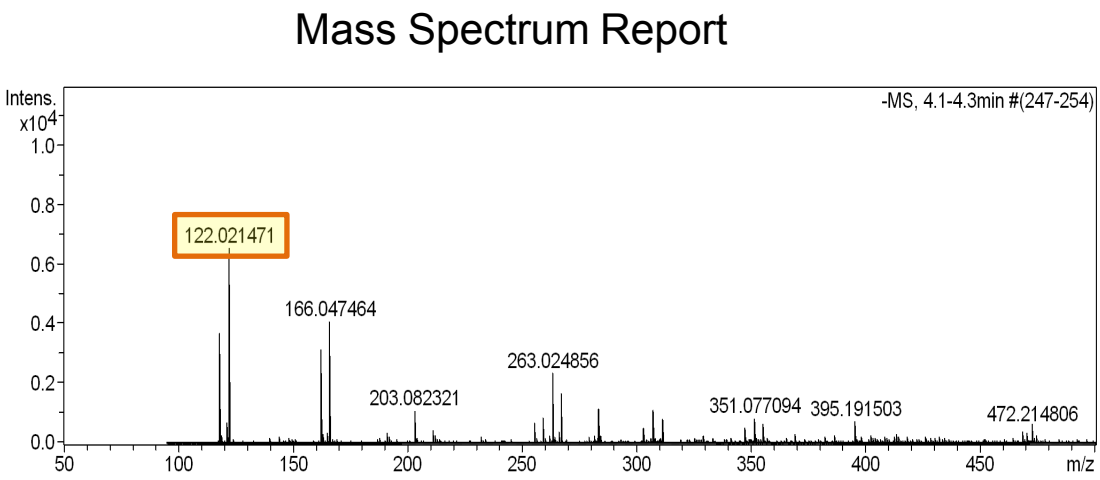


Fig. S3: Representative HPLC-MS signal of *m*/*z* 393 of buffered solutions containing ATP, CoenzymeA, compound **1**, 3-NPA and/or the fat body of *Chrysomela populi* after 1d of incubation at 30 °C.

In vivo experiments



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf
118.01428	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
121.02714	1	C 2 H 5 N 2 O 4	64.18	121.025480	-1.660	-13.720	17.4	1.5	even
122.02147	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
124.02562	1	C 2 H 6 N O 5	100.00	124.025146	-0.474	-3.821	15.8	0.5	even
162.04060	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.

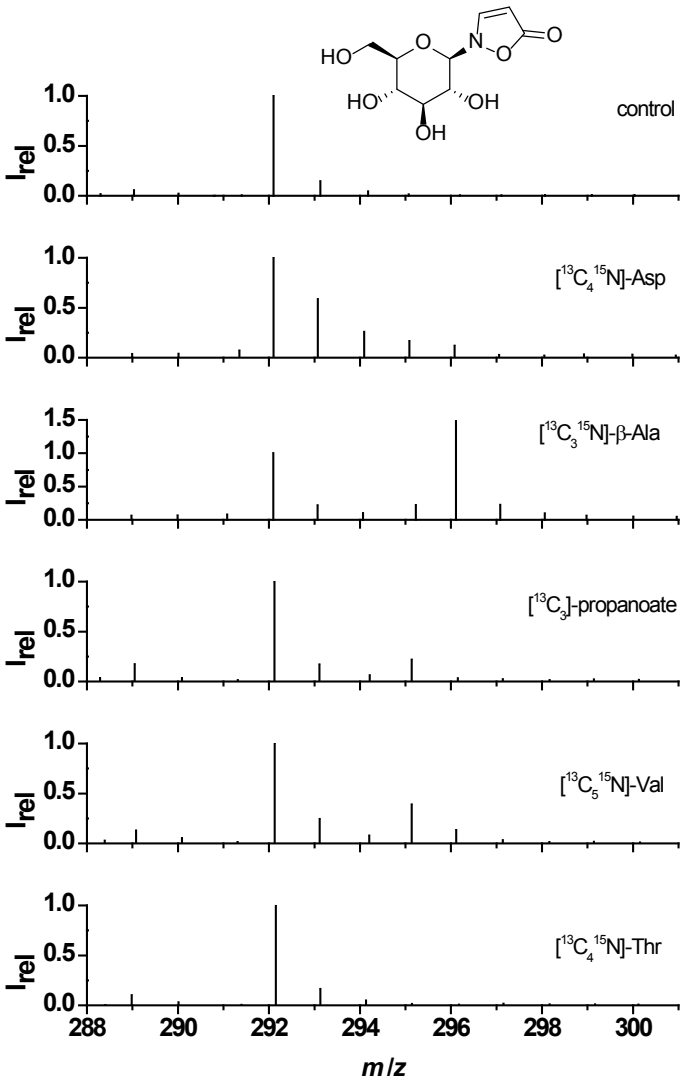


Fig. S5 Representative mass spectra of compound **1** after LC separation of larval extracts (MeCN/H_2O, 1:1) from *P. cochleariae* after feeding on different diets for 10 d; diets consisted of *B. rapa pekinensis* leaves, impregnated with KH_2PO_4/K_2HPO_4 buffered solutions of the compounds given above;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

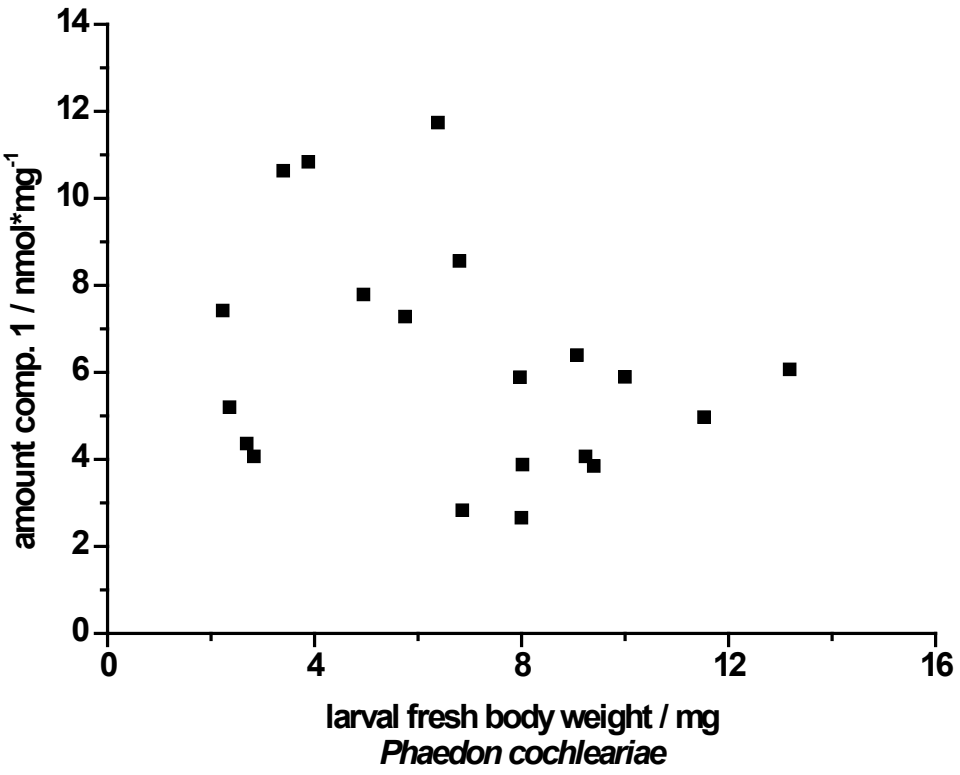


Fig. S6: Molar amount of compound **1** in *Phaedon cochleariae* per body weight, feeding on *Brassica rapa pekinensis* leafs.

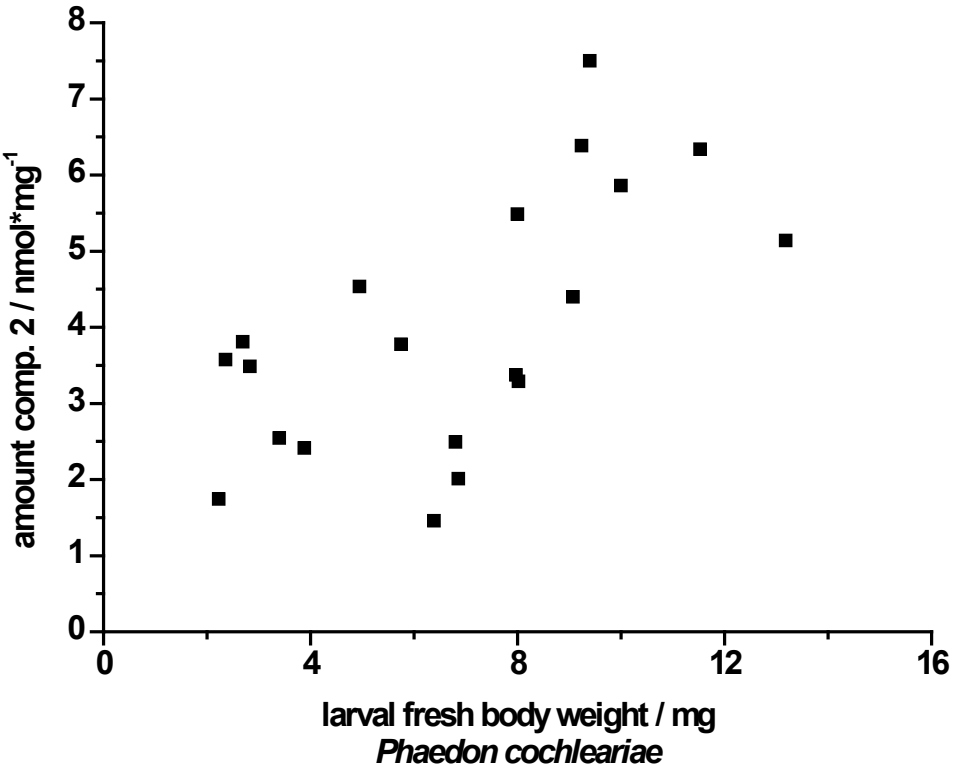


Fig. S7: Molar amount of compound **2** in *Phaedon cochleariae* per body weight, feeding on *Brassica rapa pekinensis* leafs.

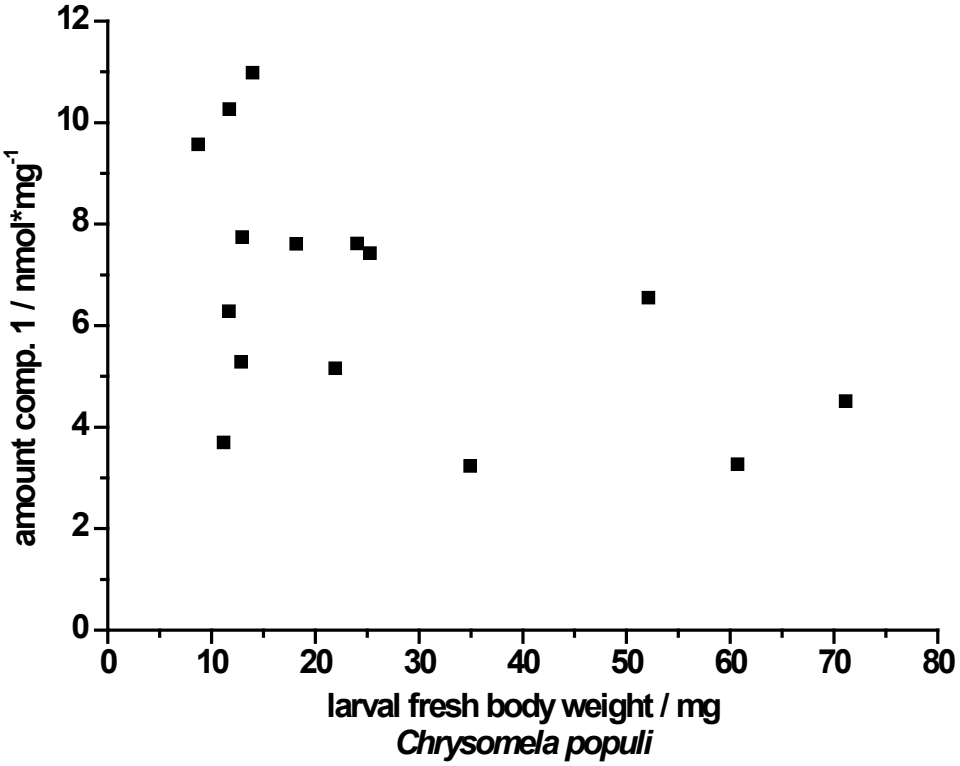


Fig. S8: Molar amount of compound **1** in *Chrysomela populi* per body weight, feeding on *Populus canadensis* leafs.

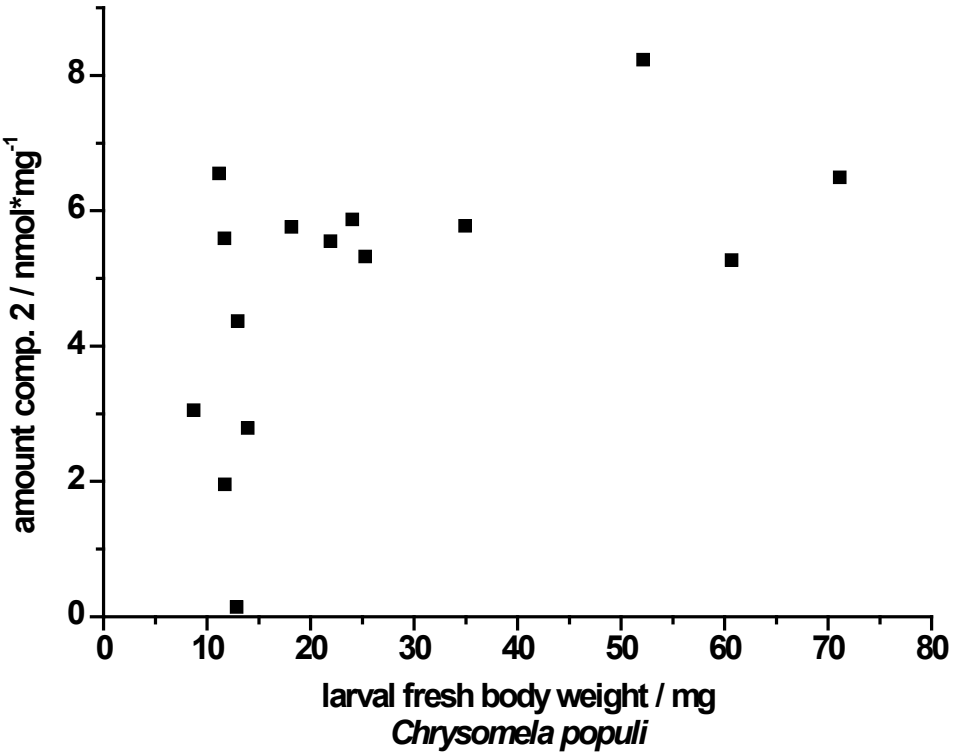


Fig. S9: Molar amount of compound **2** in *Chrysomela populi* per body weight, feeding on *Populus canadensis* leafs.

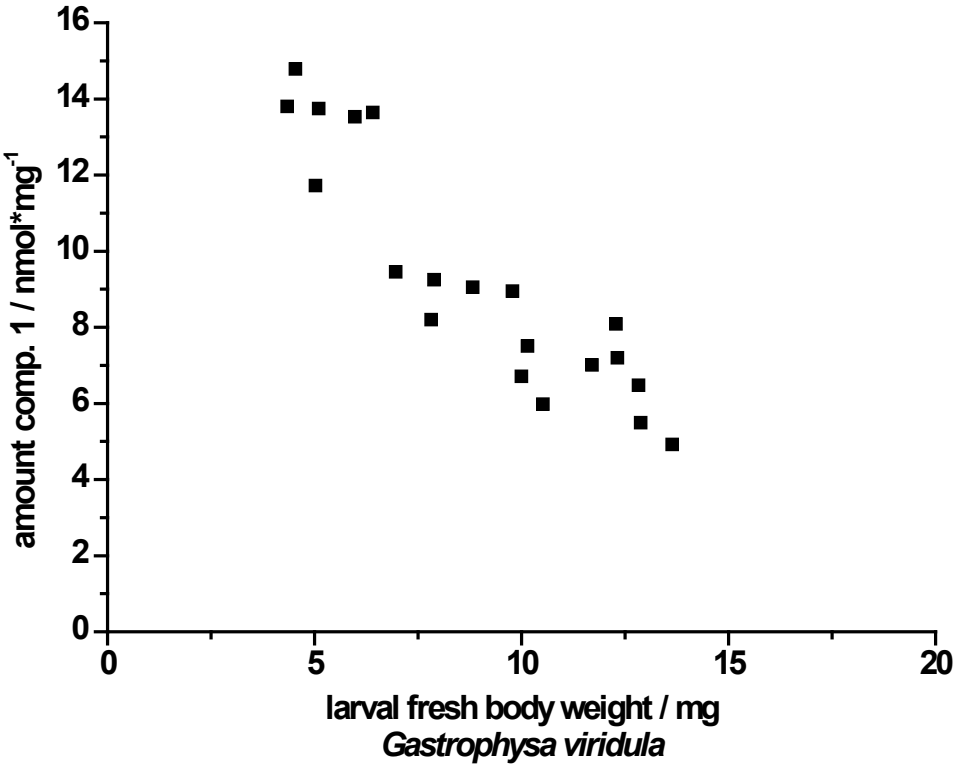


Fig. S10: Molar amount of compound **1** in *Gastrophysa viridula* per body weight, feeding on *Rumex obtusifolius* leafs.

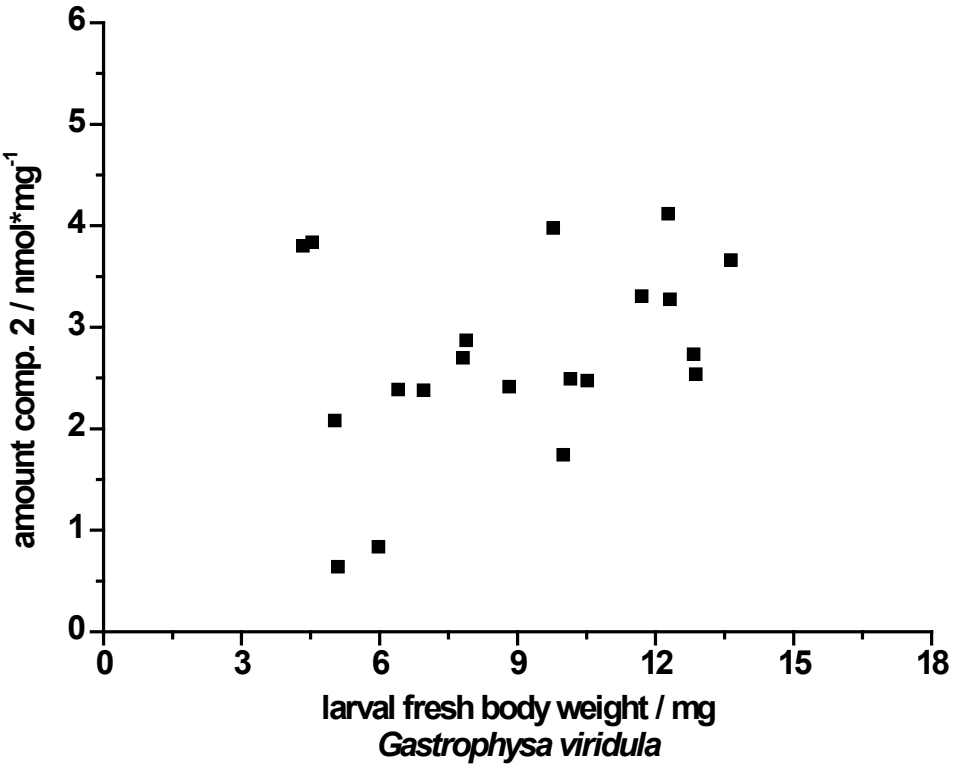


Fig. S11: Molar amount of compound **2** in *Gastrophysa viridula* per body weight, feeding on *Rumex obtusifolius* leafs.

GC-MS measurements of plant extracts

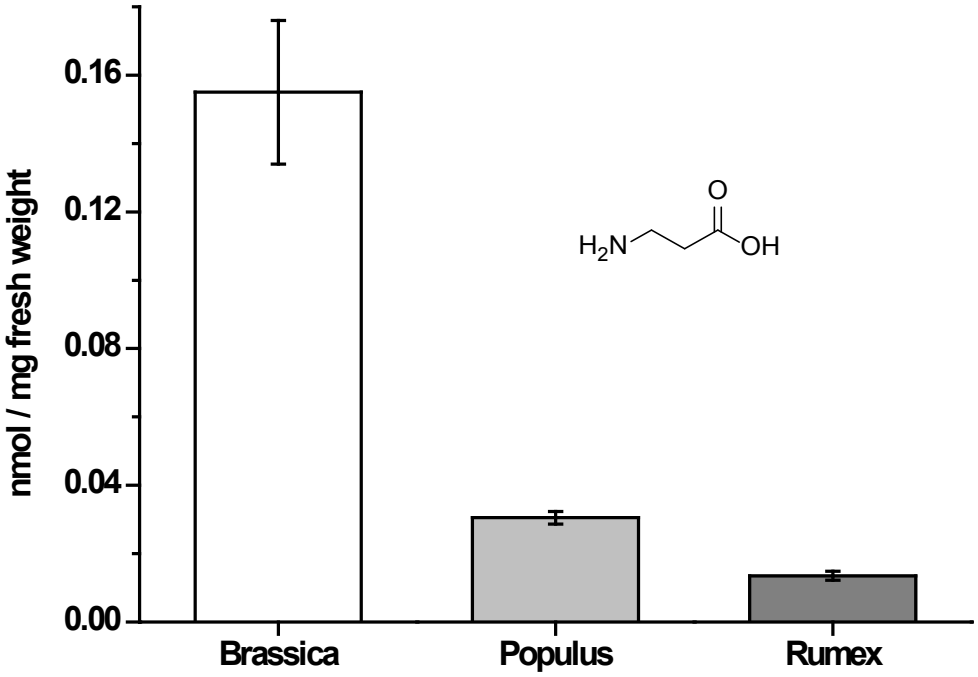


Fig. S12: Molar amounts of free β-alanine in plant leafs per mg fresh weight; n = 7 ± SD.

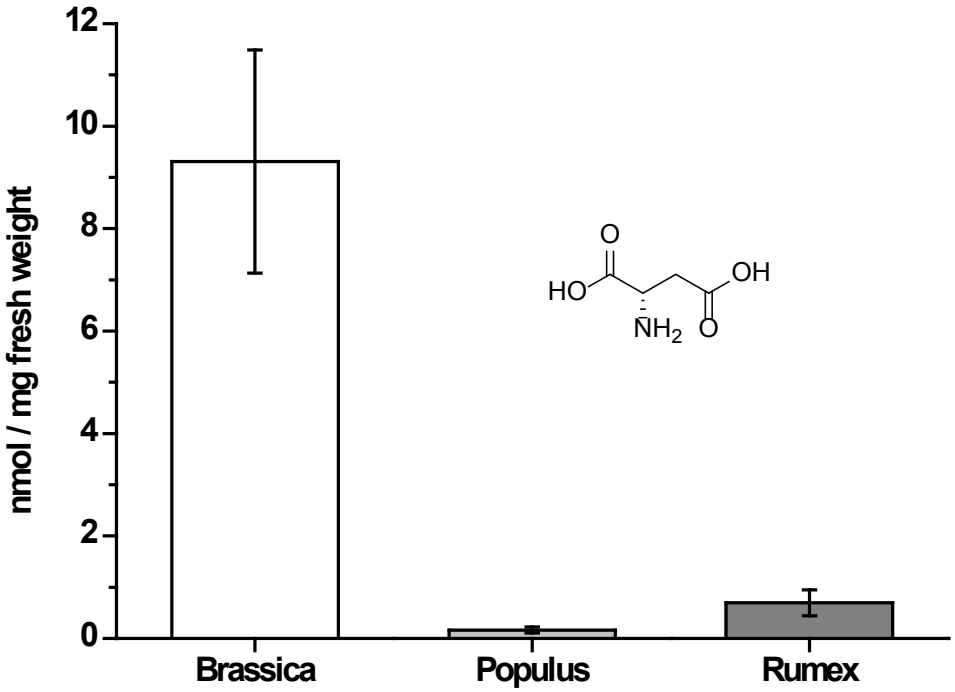


Fig. S13: Molar amounts of free aspartic acid in plant leafs per mg fresh weight; n = 7 ± SD.

Complete Publication List

All of the following citations refer to original scientific publications by Tobias Becker *et al.* in peer reviewed journals. All required information about these articles is given below:

2016

1 Pauls, G., Becker, T., Rahfeld, P., Gretscher, R., Paetz, C., Pasteels, J., von Reuss, S. H., Burse, A., Boland, W. (2016). Two defensive lines in juvenile leaf beetles; esters of 3-nitropropionic acid in the hemolymph and aposematic warning. Journal of Chemical Ecology, 42(3), 240-248. doi:10.1007/s10886-016-0684-0.

2 Becker, T., Ploss, K., Boland, W. (2016). Biosynthesis of Isoxazolin-5-one and 3-Nitropropanoic acid Containing Glucosides in Juvenile Chrysomelina. Organic & Biomolecular Chemistry, 14, 6274-6280. doi:10.1039/C6OB00899B

2015

1 Becker, T., Kartikeya, P., Paetz, C., von Reuß, S. H., Boland, W. (2015). Synthesis and photosensitivity of isoxazolin-5-one glycosides. Organic & Biomolecular Chemistry, 13, 4025-4030. doi:10.1039/C5OB00244C.

2 Becker, T., Kupfer, S., Wolfram, M., Görls, H., Schubert, U. S., Anslyn, E. V., Dietzek, B., Gräfe, S., Schiller, A. (2015). Sensitization of NO-releasing Ruthenium complexes to visible light. Chemistry-A European Journal, 21(44), 15554-15563. doi:10.1002/chem.201502091.

3 Novoselov, A., Becker, T., Pauls, G., von Reuß, S. H., Boland, W. (2015). Spodoptera littoralis detoxifies neurotoxic 3-nitropropanoic acid by conjugation with amino acids. Insect Biochemistry and Molecular Biology, 63, 97-103. doi:10.1016/j.ibmb.2015.05.013.

4 Rahfeld, P., Häger, W., Kirsch, R., Pauls, G., Becker, T., Schulze, E., Wielsch, N., Wang, D., Groth, M., Brandt, W., Boland, W., Burse, A. (2015). Glandular ß-glucosidases in juvenile Chrysomelina leaf beetles support the evolution of a host-plant-dependent chemical defence. Insect Biochemistry and Molecular Biology, 58, 28-38. doi:10.1016/j.ibmb.2015.01.003.

2013

1 Becker, T., Görls, H., Pauls, G., Wedekind, R., Kai, M., von Reuß, S. H., Boland, W. (2013). Synthesis of Isoxazolin-5-one glucosides by a cascade reaction. The Journal of Organic Chemistry, 78, 12779-12783. doi:10.1021/jo4023155.

Curriculum Vitae

Personal Data

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Current address	Hermann-Pistor-Straße 11, 07745 Jena
Email address	tbeckermgn@gmail.com

Education

11/2012-present	Max Planck Institute for Chemical Ecology
10/2010-10/2012	Master of Science, FSU Jena
10/2007-10/2010	Bachelor of Science, Universität Bremen
12/1998-06/2006	Abitur, Henfling-Gymnasium Meiningen

Military duty

07/2006-09/2007	Gebirgsjägerbataillon 233 Mittenwald

Awards

2016	Hot article in OBC
2015	2nd price at the 5th Jenaer Science-Slam competition
2012	Master of Science with an average of 1.1
2012	1st price for a presentation at a GDCh-JCF meeting
2010-2012	Fellow of the "Studienstiftung des dt. Volkes e.V."
2007-2010	Best in class in Chemistry at Universität Bremen

Presentations

Posters

Novoselov A., Becker T., Pauls G., von Reuß S.H., Boland W. (2016). Living on a poisonous diet: conjugation of fatty acids with amino acids in the gut of Spodoptera littoralis. Poster presented at 15th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE

Becker T., Boland W., Burse A., Pauls G. (2015). Synthesis and Analysis of Leaf Beetle Defensive Compounds. Poster presented at 31st ISCE Meeting, International Society of Chemical Ecology, Stockholm, SE

Becker T., Pauls G., Gretscher R. R., von Reuß S. H., Burse A., Boland W. (2014). Synthesis and Analysis of Leaf Beetle Defensive Compounds. Poster presented at 8th meeting of The Chemistry and Biology of Natural Products, Warwick, UK

Becker T., Burse A., Kugel S., Kunert M., Pauls G., Rahfeld P. (2014). Evolution of the chemical defense of juvenile and adult Chrysomelina leaf beatles. Poster presented at ICE Symposium, MPI for Chemical Ecology, Jena, DE

Rahfeld P., Pauls G., Becker T., Kugel S., Kunert M., Frick S., Boland W., Burse A. (2014). Evolution of the chemical defense of juvenile and adult Chrysomelina leaf beatles. Poster presented at SAB Meeting 2014, MPI for Chemical Ecology, Jena, DE

Oral presentations

Becker T. (2016). Biosynthesis, Chemical Synthesis and Occurrence of Hemolymph Toxins in Chrysomelina**.** Talk presented at ISCE meeting, Iguacu, BR

Becker T. (2014). Synthesis and analysis of leaf beetle defensive compounds. Talk presented at ICE Symposium, MPI for Chemical Ecology, Jena, DE

Becker T. (2014). Synthesis and analysis of leaf beetle defensive compounds. Talk presented at Naturstoffchemikertreffen, Arthur-Hantzsch-Hörsaal , Leipzig, DE

Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe.
I certify that the work presented here is, to the best of my knowledge and belief, original and the result of my own investigations, except as acknowledged, and has not been submitted, either in part or whole, for a degree at this or any other university.

_____ Jena, den 22.12.2016

Tobias Becker