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A tale of four kingdoms – isoxazolin-5-one- and 3-nitropropanoic acid-derived natural products

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Covering up to September 2016

This review reports on natural compounds that derive from the isoxazolinone ring as well as the 3-nitropropanoic acid (3-NPA) moiety. These structural elements occur in compounds that have been identified in plants, insects, bacteria and fungi. In particular, plants belonging to the family of legumes produce such compounds. In the case of insects, isoxazolin-5-one and 3-NPA derivatives were found in leaf beetles of the subtribe Chrysomelina. A number of these natural products have been synthesized so far. In the case of the single compound 3-NPA, several synthetic strategies have been reported and some of the most efficient routes are reviewed. The toxicity of 3-NPA results from its ability to bind covalently to the catalytic center of succinate dehydrogenase causing irreversible inhibition of mitochondrial respiration. As a motif that is produced by many species of plants, leaf beetles and fungi, different detoxification mechanisms for 3-NPA have evolved in different species. These mechanisms are based on amide formation of 3-NPA with amino acids, reduction to β-alanine, ester formation or oxidation to malonic acid semialdehyde. The biosynthetic pathways of 3-NPA and isoxazolin-5-one moieties have been studied in fungi, plants and leaf beetles. In the case of fungi, 3-NPA derives from aspartate, while leaf beetles use essential amino acids such as valine as ultimate precursors. In the case of plants, it is supposed that malonate serves as a precursor of 3-NPA, as indicated by feeding of ^{14}C-labeled precursors to *Indigofera spicata*. In other leguminous plants it is suggested that asparagine is incorporated into compounds that derive from isoxazolin-5-one, which was indicated by ^{14}C-labeled compounds as well. In the case of leaf beetles it was demonstrated that detection of radioactivity after ^{14}C-labeling from a few precursors is not sufficient to unravel biosynthetic pathways.

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1. Introduction

Isoxazolin-5-one and 3-nitropropanoic acid (or 3-nitropropionic acid, 3-NPA) moieties occur in a number of natural products, and all share a remarkable structural diversity. In principle, three isomeric forms of the five-membered fully unsaturated isoxazol-derived heterocycles exist. These are the 2- and 3-isoxazolin-5-one (isoxazol-5(4*H*)-one and isoxazol-5(2*H*)-one, respectively) as well as the isoxazol-5-ol isomers (Fig. 1).[1,2]

Both structural motifs are similar due the inherent O–C–C–C–N–O sequence, and mainly differ in the oxidation state of the nitrogen atom and the open-chain *vs.* ring structure.

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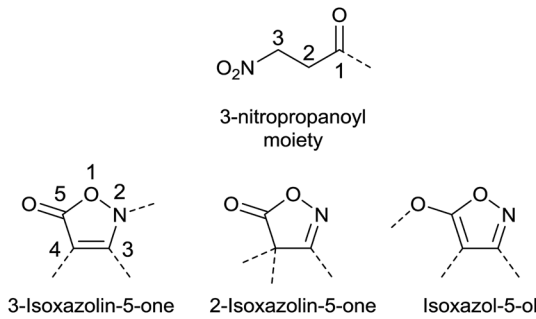

Fig. 1 Structural motifs of isoxazolin-5-one and -5-ol isomers as well as 3-nitropropanoic acid.

In biological sources, a number of natural compounds derived from isoxazolin-5-one have so far been identified in several kingdoms. Such compounds occur across the plant family of Fabaceae (Leguminosae, Papilionaceae), which contains roughly 19 500 species within 751 genera.[3] Many of these plants, *e.g. Lathyrus sativus* or *Pisum sativum*, are important food sources for livestock as well as humans. Furthermore, isoxazolin-5-ones are found in many species of leaf beetles (Chrysomelidae), belonging to the subtribe Chrysomelina. At the same time 3-NPA derived compounds occur in a number of leguminous plant and leaf beetle species. Pure 3-NPA is a toxin, causing neurodegeneration with symptoms and pathology very similar to those found in patients with Huntington's disease.[4] In some plants and beetles, 3-NPA and isoxazolin-5-one moieties are both produced by one organism. In addition, some studies revealed a fundamental relationship in the biosyntheses of the isoxazolinone heterocycle and 3-NPA in leaf beetles.[5,6] Whether in plants such relationships occur, producing both moieties, is not yet known. Since the prolonged consumption of seeds of some species of the genus *Lathyrus* (Leguminosae), *e.g. L. sativus*, causes the neuronal disease of lathyrism, many of the secondary metabolites in these plants, those deriving from isoxazolin-5-one and from 3-NPA, have been characterized in terms of neurotoxicity. These studies revealed different relationships among natural products derived from isoxazolin-5-one and 3-NPA to the disease of lathyrism, either *via* direct toxicity or upon the involvement of these natural products in the biosynthesis of other toxins, especially the neurotoxic β-*N*-oxalylamino-L-alanine (β-ODAP). Hence, attempts to genetically engineer such plants to reduce the economic damage caused by these metabolites are promising.

Within the biological kingdoms of fungi and of bacteria, natural products derived from 3-NPA and isoxazolin-5-one have also been detected. These findings are of special interest in terms of the health of human food, which is often contaminated by molds; these molds may produce the neurotoxin 3-NPA. Fermentation processes of food can also induce the production of 3-NPA by encouraging the time-dependent growth of corresponding fungi.[7] In marine organisms, benzisoxazolinones with interesting antibiotic[8] or antifouling[9] properties have been detected. Similar bioactivities were also observed in terrestrial, plant-produced isoxazolin-5-one derivatives. The motif of small nitro-acids, such as 3-NPA, was found to exhibit some activity against tuberculosis-inducing bacteria.[10]

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2. Occurrence and structural diversity

2.1. 3-NPA and derived compounds

3-Nitropropanoic acid (3-NPA) **1** was first isolated by Gorter[11] in 1920 after acidic hydrolysis of hiptagin **2**, a glucoside from the plant *Hiptage madablota* (Fig. 2).

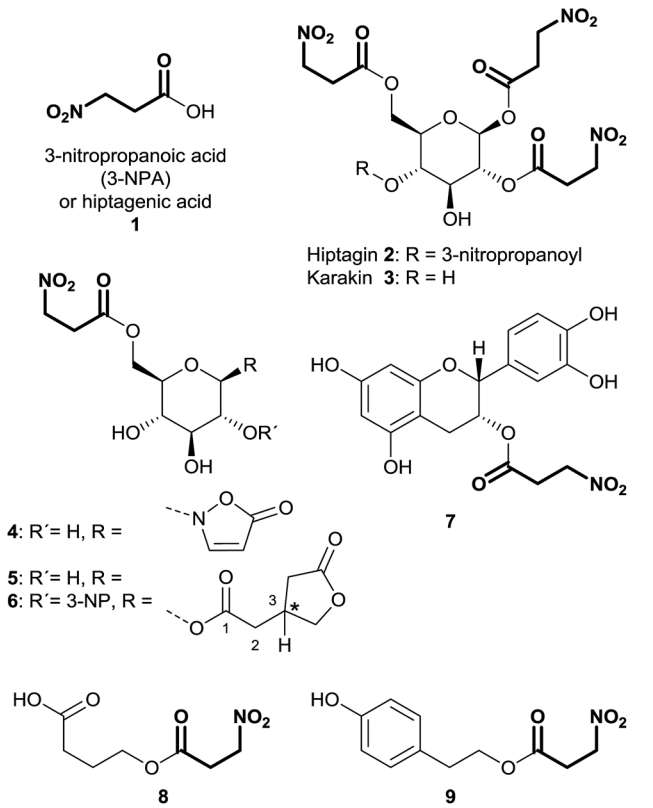

Fig. 2 3-NPA and derived compounds from diverse organisms; * = configuration not yet determined.

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Since the molecular structure of this compound was not identified, 3-NPA was designated with the trivial name "hiptagenic acid". Later, Carrie[12] isolated a substance showing an identical mixed melting point to hiptagenic acid, after applying the same hydrolytic protocol to karakin **3** (Fig. 2), which is a glucoside occurring in *Corynocarpus laevigata* fruits. The identity of 3-NPA as hiptagenic acid was finally shown by Carter and McChesney[13] as reviewed earlier by Raistrick and Stössl.[14] Since these first discoveries of 3-NPA derivatives, an increasing number of species from different kingdoms have been identified as producers of natural compounds deriving from 3-NPA. In plants, 3-NPA and its compounds have been detected in the families of Fabaceae (Leguminosae or Papilionaceae), Malpighiaceae, Corynocarpaceae, and Violaceae.[15] More than 500 species of leguminous plants belonging to at least seven genera, as shown in the cases of *Astragalus*, *Coronilla*, *Hippocrepis*, *Indigofera*, *Lotus*, *Securigera* and *Scorpiurus*, produce such compounds.[16] In particular, the amount of 3-NPA per fresh weight is higher than 100 nmol mg^{-1}, which is equivalent to more than 7% of the soluble fixed nitrogen in the shoots of some of these plants.[16,17] The chemical diversity of natural products containing 3-NPA in plants that have been identified so far is represented by the free acid itself[16,18,19] and *O*-acyl compounds of glucose and glucosides,[11,19–30] *e.g.* hiptagin **2**,[11,31] karakin **3**,[12,22] compound **4** (ref. 29 and 30) and epimers of **5** and **6**,[20] as well as from (−)-epicatechin[32] (compound **7**) and 4-hydroxybutanoic acid **8** (ref. 33) (Fig. 2). For an overview of the occurrence of glucosides derived from 3-NPA see R. Parry *et al.*[34] It is important to note that the structures of compounds **5** and **6** were originally assigned as 1-*O*-acyl glucosides[20] as shown in Fig. 2, while aliphatic *O*-glucosides were shown for these compounds in the corresponding reviews.[34,35] In the case of ref. 35 no stereochemistry was addressed at all, and thus the structure of hiptagin **2** was not assigned correctly in this review either. The absolute stereochemistry of the epimers of **5** and **6** was not assigned, although there was no indication of diastereoisomers in these isolates.[20] The aglycon, isolated from *Astragalus canadensis* samples, 3-hydroxymethyl glutaric acid, was optically inactive.[36] A racemization of this free acid can proceed *via* hydrolysis, followed by re-lactonization or *via* direct intramolecular attack of the free carboxyl function (Fig. 3).

Hence, even though the aglycones ((*R*)- and (*S*)-3-HGAGL) occur as racemates, selective *O*-acylation of a glucosyl donor with an activated form of one of the possible enantiomers of 3-hydroxymethyl glutaric acid γ-lactone can, *via* enzymatic catalysis, lead to pure diastereomers of compounds **5** and **6** at the 3-position of the lactone, as reported by Benn *et al.*[20]

In fungal species, free 3-NPA has been identified in *Aspergillus*, *Arthrinium*, *Diaporthe* and *Penicillium* genera.[15,37–39] Furthermore, the ester compound 4-hydroxyphenethyl 3-nitropropanoate **8** was isolated from endophytic *Phomopsis* sp. PSU-D15 (Fig. 2).[40]

3-NPA derived compounds were also detected in leaf beetle species (Coleoptera: Chrysomelidae), belonging to the subtribe Chrysomelina (Chrysomelini), *e.g. Phaedon cochleariae* and

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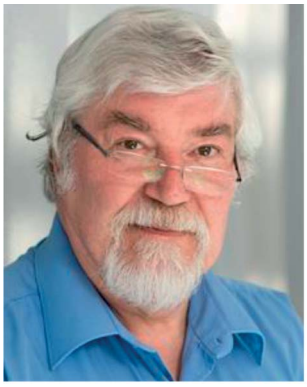

Wilhelm Boland studied Chemistry and received his PhD from the University of Cologne in 1978. He was a Research Associate at the Institute of Biochemistry (University of Cologne) until 1987 and moved then to the University of Karlsruhe as a Professor for Organic Chemistry. In 1994 he became Full Professor for Bioorganic Chemistry at the University of Bonn. In 1997 he joined the Max Planck Society and became Director of the Department of Bioorganic Chemistry in the Max Planck Institute for Chemical Ecology in Jena. Professor Boland was the 1st Simeone-Silverstein lecturer (1995) of the International Society of Chemical Ecology and president of the society from 2008 to 2009.

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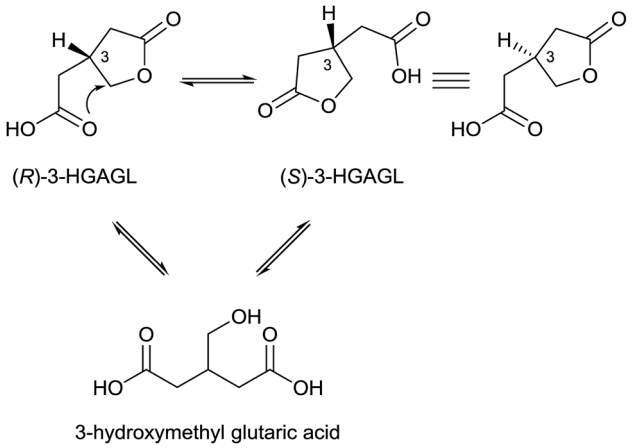

Fig. 3 Suggested equilibria between (*R*)- and (*S*)-3-hydroxymethyl glutaric acid γ-lactone (3-HGAGL) in solution.

Chrysomela populi (Fig. 2).[41–46] In these organisms, compound **4** is present in all life stages of the insect, occurring as a major secondary metabolite in the eggs, the larval hemolymph, the pupae and the adult hemolymph and their defensive secretions.[6,41–45,47–51] Additionally, in adult secretions of the four leaf beetle species *Gastrophysa atrocyanea*, *Plagiodera versicolora distincta*, *Chrysomela vigintipunctata costella*, and *Gastrolina depressa*, belonging to the subtribe Chrysomelina, diverse esters of glucose containing 3-NPA and isoxazolin-5-one motifs were indicated by TLC and their structures were determined by HPLC, MS and NMR analyses.[46] These findings describe structures without the isoxazolin-5-one heterocycle, bearing up to three 3-NPA moieties in different positions on the sugar.[46]

In the eggs, concentrations around 10^{-2} M (M = mol L^{-1}) of compound **4** were reported for *Gastrophysa viridula*, *Chrysomela tremulae* and *C. populi*.[44] The same concentration ranges were determined for this compound in the larval hemolymph,[41] and show increasing tendencies with respect to the larval body weight.[5,41]

To gain a comprehensive picture of the occurrence of 3-NPA and its derivatives in natural samples, it is of interest whether 3-NPA can be found only in its unaltered free form, or if it is found as a derivative, *e.g.* an ester as shown in many examples in Fig. 2. In leaf beetles, the content of free 3-NPA in the hemolymph was detected only in low amounts that were probably caused by the immediate hydrolysis of ester compounds, *e.g.* **4**, in fresh natural samples. In adult secretions, the free toxin was detected, probably occurring due to hydrolysis after transport of the corresponding glucosides from the hemolymph. In contrast, studies concerning the formation 3-NPA in microbiota often describe the production of the free toxin. In plants, as shown above, mainly the free acid and the *O*-acyl compounds of 3-NPA and glucosides or other alcohols have been described. Nevertheless, in many studies it remains unclear whether 3-NPA occurs as a derivative, the free toxin or in both forms.

In order to detect free 3-NPA, several screening methods have been established, mainly to analyze toxic contaminations (such as 3-NPA) in food sources, caused by microbiota.[52–55] In MS analyses, nitro alkanes exhibit characteristic ions, *e.g.* NO_2^- (*m*/*z* 46), allowing the application of precursor ion scans and other established MS techniques.[56,57] These analyses proceed under mild ionization conditions, such as atmospheric pressure chemical ionization (APCI), and can be coupled with previous LC separation, which is necessary for the detection of most of the 3-NPA derivatives from natural samples that have been described so far. In addition to their specific behavior in mass spectrometry, 3-NPA derivatives show characteristic NMR signals. The two methylene groups in the 3-NPA moiety show solvent dependent diagnostic signals around δ_H 3.02 (C-2-**H**$_2$, triplet, $^3J_{2,3}$ = 5.8 Hz), δ_C 31.7 (**C**-2) ppm and δ_H 4.71 (C-3-**H**$_2$, triplet, $^3J_{2,3}$ = 5.8 Hz), δ_C 70.7 (**C**-3) ppm in the case of ester **4** in D_2O.[41,45,46,58]

2.2. Isoxazolin-5-one derivatives

Natural products of the described class mainly occur as derivatives of 3-isoxazolin-5-one, rather than the 2-isoxazolin-5-one or the isoxazolol isomers. So far, 3-isoxazolin-5-one derivatives have been identified in plants,[59–73] leaf beetles (Chrysomelina),[6,41–51] bacteria of the genus *Streptomycetes*[74–76] as well as the cold-water sponge *Geodia barretti*,[9] the fungus *Fusarium larvarum*[77] and the marine bacterium *Pseudoalteromonas flavipulchra* JG1.[8] The structural diversity of naturally occurring isoxazolin-5-one compounds varies from derivatives of amino acids (TAN-950A **10**, BIA **11** and ACI **12**),[59,74–76] peptides (γ-glu-AEI **13** and γ-glu-BIA **14**),[59,61,62,64,78] ethylamine (AEI **15**),[79] carboxylates (CMI **16** and **17**)[59,70,79] and cyanoethane (BIP or CEI **18**)[62,68,71] to glucosides, *e.g.* compounds **4**,[6,29,30,41,42,44–51] **19** (ref. 41, 44, 45 and 67) and **20** (ref. 65 and 69) (Fig. 4). Furthermore, benz-3-isoxazolin-5-ones such as parnafungin B1 **21**,[77] bromobenzisoxazolone barettin **22** (ref. 9) and *N*-hydroxybenzoisoxazolone **23** (ref. 8) have been isolated. Common abbreviations and names used for the mentioned compounds in the cited literature are presented in Fig. 4.

In *Lathyrus odoratus* (Leguminosae), it was shown that single 3-isoxazolin-5-one derivatives **11–13**, **15** and **18–20** occur in contents of up to >1% of the dry weight. Compound **19** is produced in amounts of up to 2.64 nmol mg^{-1} fresh weight in 7 day-old seedlings of *L. odoratus*.[65] In general, as expected, the amounts of these compounds show spatial and temporal variations in the plant.[65]

In leaf beetles, similar trends were observed, as compounds **4** and **19** occur in amounts of 2 to 12 nmol mg^{-1} body weight in *Phaedon cochleariae*.[5,41] Analogously, these compounds were detected in millimolar concentrations in the eggs of these insects.[44]

Within compound **4**, both of the discussed moieties, 3-NPA and 3-isoxazolin-5-one, occur in the same molecule. This substance has been identified as a major component of secretions and hemolymph of leaf beetles,[6,41,42,44,45,47,50,51] as well as of two species of *Astragalus*[29,30] plants (Fabaceae). Furthermore, various plants from the Fabaceae family produce 3-isoxazolin-5-one and 3-NPA derivatives. Thus, it is of interest to know in which other species, especially in the legume family, both moieties can be detected at the same time. To study the occurrence of 3-isoxazolin-5-one derivatives, UV-absorption spectra were measured.[59,80] These analyses revealed strong absorption of the 2-substituted

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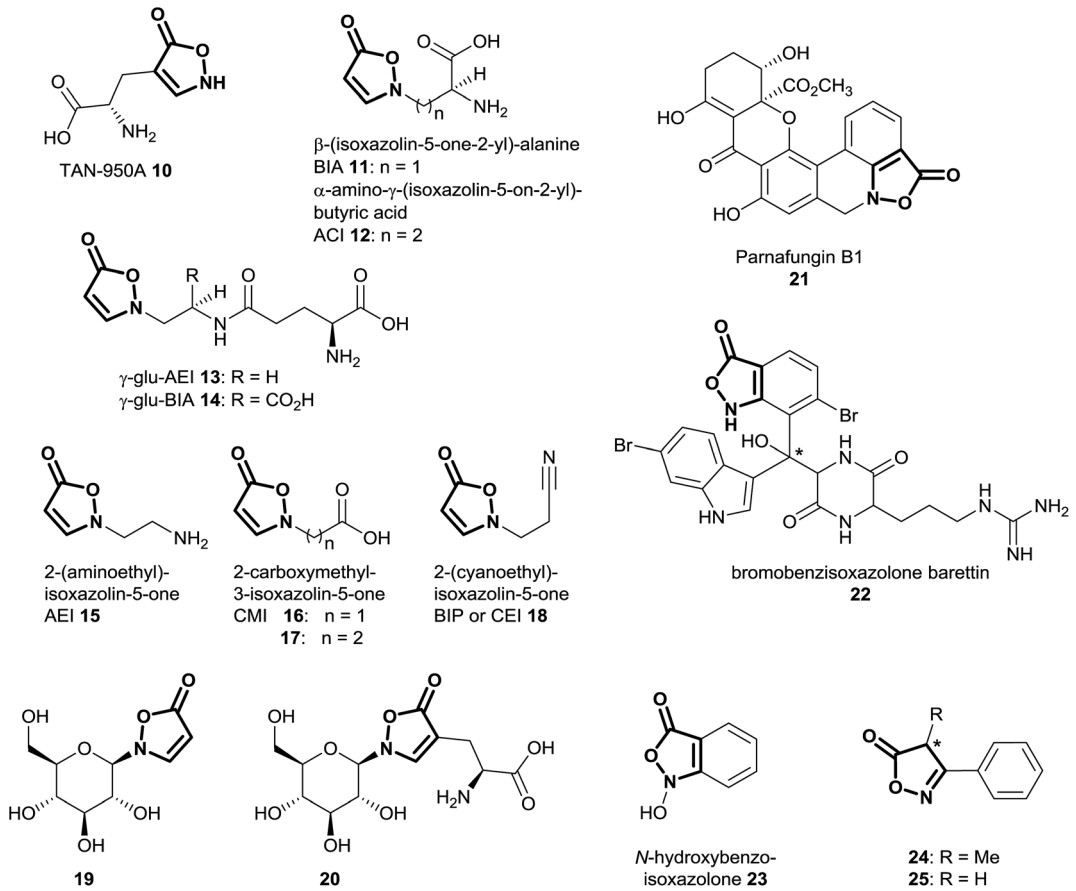

Fig. 4 Structures of naturally occurring isoxazolin-5-one derivatives with highlighted isoxazolin-5-one structure; * = stereochemistry is under investigation; compounds **24** and **25** were indicated only by GC-MS (see text).

derivatives of the heterocycle around 260 nm,[59,80] with extinction coefficients above 10^4 L mol^{-1} cm^{-1}.[58,73] These properties indicate the occurrence of putative 3-isoxazolin-5-one compounds in natural samples, as shown by capillary zone electrophoresis[62] or HPLC-UV[81]-based analyses.

If the isolated amounts or concentrations of the compounds in natural samples are sufficient, additional NMR data can be acquired.[41] Typical NMR signals of 3-isoxazolin-5-one derivatives occur around δ_H 5.52 (C-4-**H**, doublet, $^3J_{3,4}$ = 3.7 Hz), δ_C 91.7 (**C**-4) ppm and δ_H 8.47 (C-3-**H**, doublet, $^3J_{3,4}$ = 3.7 Hz), δ_C 155.0 (**C**-3) ppm as well as δ_C 174.7 (**C**-5) ppm, in 2-substituted, 3,4-unsubstituted compound **4** in D_2O.[41,45,46,58] The benzisoxazolinone compound **22** shows carbon shifts at δ_C 170.3 (**C**-5) , 137.7 (**C**-3) and 138.7 (**C**-4) ppm in d^6-DMSO.[9] In compound **21**, typical carbon shifts occurred at δ_C 167.3 (**C**-5), 155.6 (**C**-3) and 109.7 (**C**-4) ppm (d^6-DMSO).[77]

There is some indication from GC-MS analyses of methanolic extracts of species belonging to the endophytic fungal genus *Xylaria* that 3-phenyl-4-methyl-2-isoxazolin-5-one **24** (Fig. 4) may be a component of the secondary metabolites.[82] For this substance, no isolation and structure elucidation was carried out, and thus the absolute stereochemistry of the putative structure is unknown.[82] The presence of the 2-isoxazolin-5-one derivative was indicated only by comparison with the NIST compound library (95% identity in the EI-mass spectra).[82] Analogously, indications for the occurrence of 3-phenyl-2-isoxazolin-5-one **25** in the Chinese traditional medicine prescription "Xiaoxuming" decoction (aqueous boiling extraction of herbs) were obtained by similar GC-MS-based analyses.[83] Since these compounds are used particularly as synthetic intermediates,[84] the occurrence of such 2-isoxazolin-5-one derivatives in *Xylaria* and related species should be investigated by additional analytical techniques, *e.g.* HPLC-(HR)MS as well as NMR.

In analyses applying GC-MS methods using hydroxylamine- and trimethylsilyl (TMS)-derivatization of human urine samples, acetoacetic acid has been observed to form TMS derivatives of 3-methyl-isoxazol-5-ol and 3-methyl-3-isoxazolin-5-one.[85] Hence, these compounds were identified as artefacts, formed by the application of hydroxylamine to the natural samples.[85] In a further publication, a similar observation has been described including the isolation and characterization by ^{13}C NMR of the untreated 3-methyl-2-isoxazolin-5-one after reaction of hydroxylamine and acetoacetic acid.[86]

3. Biosynthesis of 3-isoxazolin-5-one and 3-NPA derivatives

3.1. Microbiota

Early publications on the biosynthesis of 3-NPA from fungal origin describe the incorporation of aspartate into 3-

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nitropropanoic acid.[87] By applying radioactive [4-^{14}C]-DL-aspartic acid to *Penicillium atrovenetum*, it was demonstrated that 2% is incorporated, showing 96% of the labeling at the C-1 position in 3-NPA **1**.[87] Later, it was observed that the L-isomer of aspartic acid is the preferred precursor.[88] Hence, it could be demonstrated that other compounds associated with the tricarboxylic acid cycle, *e.g.* pyruvate and acetate, are also precursors of 3-NPA in this fungus.[89] In contrast to the findings in leaf beetles, β-alanine failed to be incorporated into compound **1** after the application of radioactive precursors.[87] Additional experiments indicated that aspartate serves as a nitrogen source for the biosynthesis of 3-NPA in *P. atrovenetum*.[88] Using [2-^{13}C,^{15}N]-DL-aspartic acid, Baxter *et al.* showed by NMR analyses that the C–N bond in aspartate is conserved upon 3-NPA biosynthesis in *P. atrovenetum*.[90] Further experiments on the biosynthesis of 3-NPA in this fungus resulted in the confirmation of L-nitrosuccinate **26** as an intermediate within this metabolic pathway, which indicates that the pathway proceeds *via* direct oxidation of the nitrogen atom in the precursor L-aspartic acid (Fig. 5).[37,91] This oxidation is probably a stepwise process, where the consumption of dioxygen produces *N*-hydroxy intermediates, as indicated by the application of $^{18}O_2$ to the fungus.[37]

Furthermore, it was indicated that compound **26** decarboxylates spontaneously under physiological conditions and hence an enzymatically catalyzed transformation to 3-NPA is probably not required.[92]

In vitro studies with enzymes from *Streptomyces* and other bacteria also showed that 3-NPA is formed *via* oxidation of L-aspartate.[93] In this case the reaction is catalyzed by a flavin-dependent oxidoreductase FzmM, which is involved in the biosynthesis of fosfazinomycin.[93] Thus, 3-NPA occurs as a putative intermediate within the biosynthesis of this natural product in bacteria.

3.2. Leaf beetles

First experiments on the biosynthesis of isoxazolin-5-one and 3-NPA derivatives in the case of leaf beetles were carried out with adults of the species *Chrysomela tremulae* (Chrysomelina).[6,51] For this purpose, [$^{14}C_4$]-aspartate covered leaves were presented to adults of *C. tremulae* for one week.[6] After this time, radioactivity was measured in 3-isoxazolin-5-one glucosides **4** and **19**, which are the major components of the defensive adult secretions. In the 3-NPA ester **4**, a higher specific activity was determined and after acidic hydrolysis, the resulting free glucose showed negligible radioactivity. As a consequence, it was concluded that both moieties, 3-NPA as well as the 3-isoxazolin-5-one heterocycle, derive from aspartate as a precursor, analogous to the biosynthesis of 3-NPA in fungi. Since the natural products **4** and **19** were not detected in the host plants of the insects, these results further indicated that these secondary metabolites are *de novo* produced by the insects. No information about the intactness of the incorporation or corresponding intermediates in the pathway was provided in this study. Furthermore, low relative incorporations of aspartate into compounds **4** and **19** of around 0.016% were determined. These adult defensive compounds were also detected in high amounts (mM concentrations) in the larval[41] and adult hemolymph as well as the pupae. Consequently, the feeding experiments were repeated and expanded using leaf beetle larvae, which are easier to rear and treat, by applying stable isotope labeled compounds.[5] These studies revealed an incorporation of randomly re-assembled fragments of aspartate into compounds **4** and **19**. In contrast, the intact incorporation of the essential amino acid valine was observed. Further investigations revealed that the biosynthetic pathway proceeds *via* propanoic acid (in the propanoyl-CoA form) as well as β-alanine. This pathway has been described previously,[94] proceeding *via* oxidation of propanoyl-CoA to form acrylyl-CoA,[94,95] followed by addition of ammonia or further oxidation to malonate semialdehyde, which can be transaminated.

Whether only one or both of these pathways occur in leaf beetles has not yet been investigated. Nevertheless, the last two reactions (addition of ammonia as well as transamination) could provide β-Ala. The latter compound is then step-wise oxidized at the nitrogen atom to form compound **27** and the corresponding oxime **29** (*E*/*Z*) or 3-NPA **1**, probably *via* the dihydroxylated intermediate **28**. Intermediates **27** and **29**, as well as 3-NPA, were synthesized as [^{13}C,^{15}N]-labeled compounds and applied *in vivo* to the beetles, analogous to commercial multiple stable-isotope-labeled aspartate, valine, propanoic acid and β-alanine. The oxime **29** (or the respective CoA-ester) can be cyclized to produce the unsubstituted 3-isoxazolin-5-one **30** (and its tautomers), which was shown to be transformed into compound **19** by reaction with α-UDP-glucose in ^{1}H NMR assays. As a last step, 3-NPA is activated under consumption of ATP to form the corresponding *S*-CoA ester, which is transesterified to form the nitroester **4** (Fig. 6).[5]

In agreement with the previous results,[6] the relative incorporation of aspartate was determined to be 0.019%,[5] while the incorporation of valine was 8.1 fold higher. In the case of β-alanine and propanoic acid, the relative incorporations were 17.4 ± 11.2% and 2.3 ± 1.5%, respectively.[5] These results[5,6] reveal the fundamental relationship of the isoxazolin-5-one biosynthesis to the biosynthesis of 3-NPA in leaf beetles. In some species, the concentration of the 3-NPA moiety increases with the larval body weight, while the concentration of the glucoside **19** is more or less constant.[5,41] These observations indicate tight control of different enzymes involved in the β-alanine *N*-oxidation, allowing selective oxidation of the amino

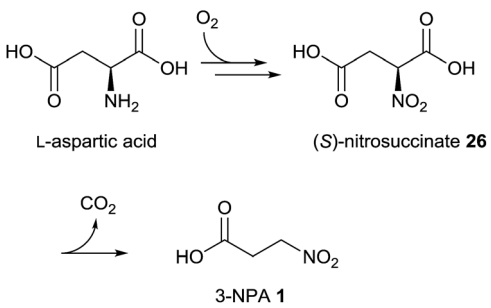

Fig. 5 Biosynthetic pathway of 3-NPA in *P. atrovenetum*.[37]

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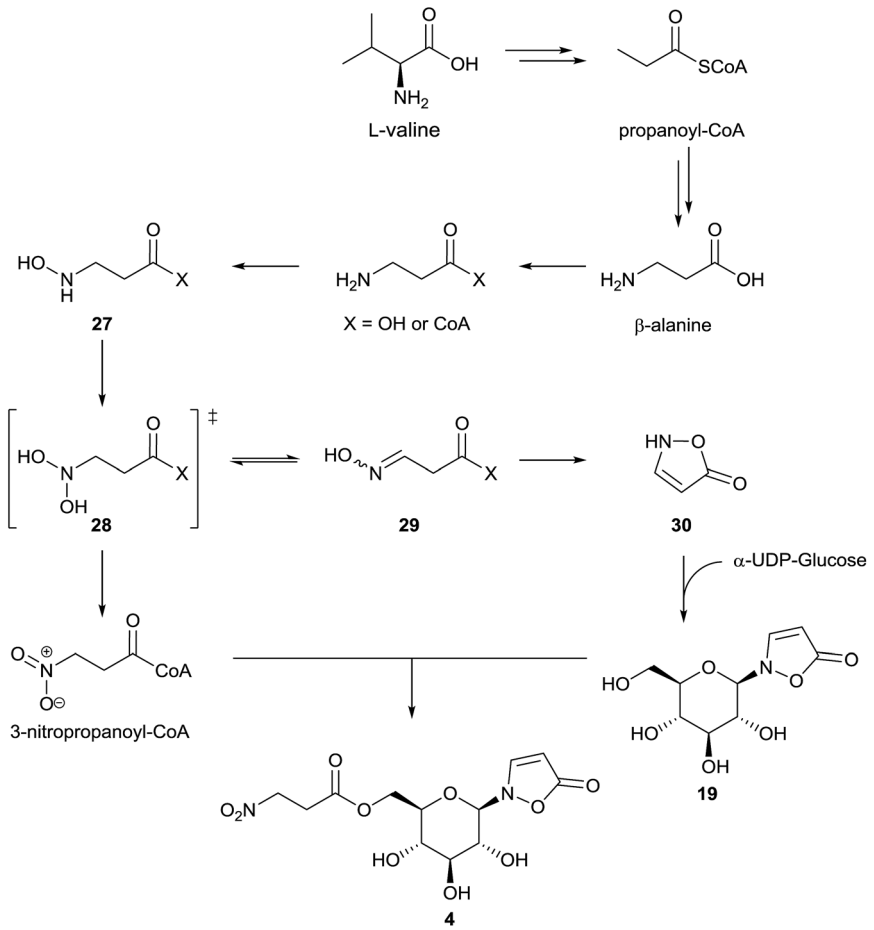

Fig. 6 Proposed biosynthetic pathway of compounds **4** and **19** in Chrysomelina.

group to form either the oxime (by enzyme group I) or the nitro group (by enzyme group II).

Recently published results[5] on the biosynthesis of compounds **4** and **19** in Chrysomelina relate to juvenile beetles. The extent to which the biosynthesis in the adult beetles equals the biosynthesis of these compounds in the larval stage has not yet been investigated; it is likely that identical pathways exist, given the identical genotype in all life stages of one organism.

3.3. Plants

To study the biosynthesis of 3-NPA in the leguminous plant *Indigofera spicata* (creeping indigo), several putative radioactive labeled precursors were applied.[96] In these experiments, a relative incorporation of [2-^{14}C]-malonate of between 0.017 and 0.047% was detected, while aspartate failed as a precursor, shown by the absence of radioactivity in 3-NPA after the plants were fed on ^{14}C-labeled aspartic acid.[96] Consequently, [2-^{14}C]-malonyl-monohydroxamic acid **32** as a putative malonate-derived intermediate was synthesized and applied to the plant, resulting in relative incorporation rates of between 0.018 and 0.081%.[96] Therefore, a biosynthetic pathway for the 3-NPA production in *I. spicata via* malonate, malonyl monoamide **31**, *N*-hydroxy-β-alanine **27** and malonyl monohydroxamate **32** was suggested (Fig. 7, right pathway).[96]

In addition to the described findings, the position of incorporation within the C_3-unit of 3-NPA was determined with different results. These experiments indicate that incorporation occurs in the (expected) C-2 position as well as C-3, but not the C-1 position.[96] A positive incorporation into positions other than C-2 indicate random fragmentation and the re-assembly of malonate due to diverse metabolic reactions within the plant, as exemplified in the biosynthesis of 3-NPA in leaf beetles *via* randomly re-assembled aspartate.[5] In addition, the determined relative incorporations were rather small, being comparable to the (indirect) incorporation of aspartate into compounds **4** and **19** in leaf beetles.[5,6] Thus, a direct biosynthetic pathway deriving from non-malonate-derived metabolites, *e.g.* valine and propanoate as well as β-alanine, may not be excluded from the presented results of this[96] study. In bacterial[97] as well as archaeal[98] species, it was shown that malonate can be reduced to form malonate semialdehyde **33**. As this substance is a precursor of β-alanine[94] in diverse organisms, it is of interest whether the observed malonate incorporation is due to the subsequent formation of β-Ala *via* transamination of malonate semialdehyde **33** with *e.g.* glutamate (Fig. 7, left pathway).

Since aspartate was not a precursor of 3-NPA in creeping indigo, the involvement of symbiotic microbiota in the biosynthesis of this toxin from primary metabolites seems

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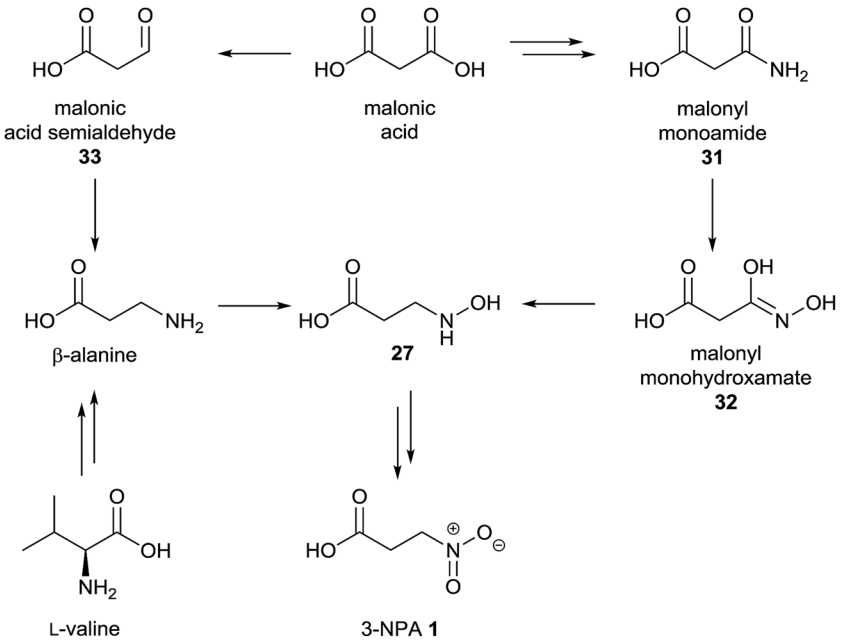

Fig. 7 Proposed biosynthetic pathway of 3-NPA **1** in *I. spicata*, adapted from Candlish *et al.*[96] (right) and alternative pathway (left).

unlikely. Nevertheless, 3-NPA occurs in a range of different plant species that may show different metabolic pathways for 3-NPA biosynthesis, and thus some contribution of microorganisms in the 3-NPA production cannot be ruled out. Extracts of a number of endophytic fungi contained 3-NPA, indicating microbiota play a role in the total production of this compound.[99]

Investigations on the biosynthesis of the isoxazolin-5-one moiety of different natural products were carried out in some *Lathyrus*, *Pisum*, *Leucaena* (Leguminosae) and *Citrullus* (Cucurbitaceae) species (Fig. 8).

In vivo studies in *L. odoratus* plants using [$^{14}C_4$]-asparagine revealed the positive incorporation into compounds BIA **11**, ACI **12**, CEI **18** and **20**.[100] Photohydrolysis experiments indicated that the incorporation of [$^{14}C_4$]-asparagine occurs to different extents in the side-chain and the heterocycle.[100] In *L. sativus*, it was demonstrated that the reaction of *O*-acetyl-L-serine (OAS) with isoxazolin-5-one **30** results in the formation of BIA **11**.[101] This reaction was supposed to be catalyzed by cysteine synthase isolated from the plant.[102] Furthermore, it was shown that BIA **11** is a precursor in the biosynthesis of the plant toxins L-α,β-diaminopropanoic acid and β-*N*-oxalyl-L-α,β-diaminopropanoic acid as well as γ-glu-AEI **13** and γ-glu-BIA **14**.[64,103–105] Later, it was demonstrated that enzyme isolates of cysteine synthases from *L. sativus* as well as *L. odoratus* can also catalyze the reaction of isoxazolin-5-one and OAS to form TAN-950A **10**, the 4-substituted isomer of BIA **11**, which is a structural motif in the glucoside **20**.[106] The glucoside **19** is formed *via* the reaction of isoxazolin-5-one **30** and α-UDP-glucose. This transformation is catalyzed by enzyme extracts from *L. odoratus* as well as *P. sativum*.[107] NMR experiments showed that this reaction also occurs in leaf beetles.[5] After reaction of **19** with OAS, compound **20** is formed. This reaction is catalyzed by extracts from *L. odoratus*, *P. sativum*, *Citrullus vulgaris* and *Leucaena leucocephala*.[108] *In vitro* assays using enzyme extracts from *L. odoratus* indicated that the biosynthesis of ACI **12**, the homologue of BIA **11**, proceeds *via* isoxazolin-5-one as well as *S*-adenosyl-L-methionine as substrates.[109,110]

As described in previous chapters, the isoxazolin-5-one and 3-NPA moieties occur in *Astragalus* species in the same molecule, as found in compound **4**. Whether there is a relationship between the metabolic pathways of both moieties, similar to the biosynthesis of these motifs in leaf beetles, is not yet known.

Additional analyses may shed light on related topics such as the incorporation of applied precursors into the isoxazolin-5-one and 3-NPA motifs of the natural products. Furthermore, the genetic, transcriptomic and proteomic basics of the 3-NPA biosynthesis should be addressed in important food plants such as *L. sativus*.[111–113]

4. Chemical synthesis and properties of 3-NPA and 3-isoxazolin-5-one derivatives

4.1. Syntheses of 3-NPA

An almost quantitative method to realize the synthesis of 3-NPA **1** was described based on the oxidative cleavage of the corresponding β-lactam **34** conducted by a dioxirane (Fig. 9a).[114]

An alternative way to achieve the synthesis of 3-NPA **1** proceeds *via* oxidation of the analogous aldehyde 3-nitropropanal **35**, starting from commercial acrolein **36** (Fig. 9b).[115]

In order to realize a tailored introduction of stable heavy isotopes into 3-NPA **1** to study biosynthetic aspects of this component, a three step synthesis in which 2-chloroethanol **37** was used as the starting material yielded intermediate 3-

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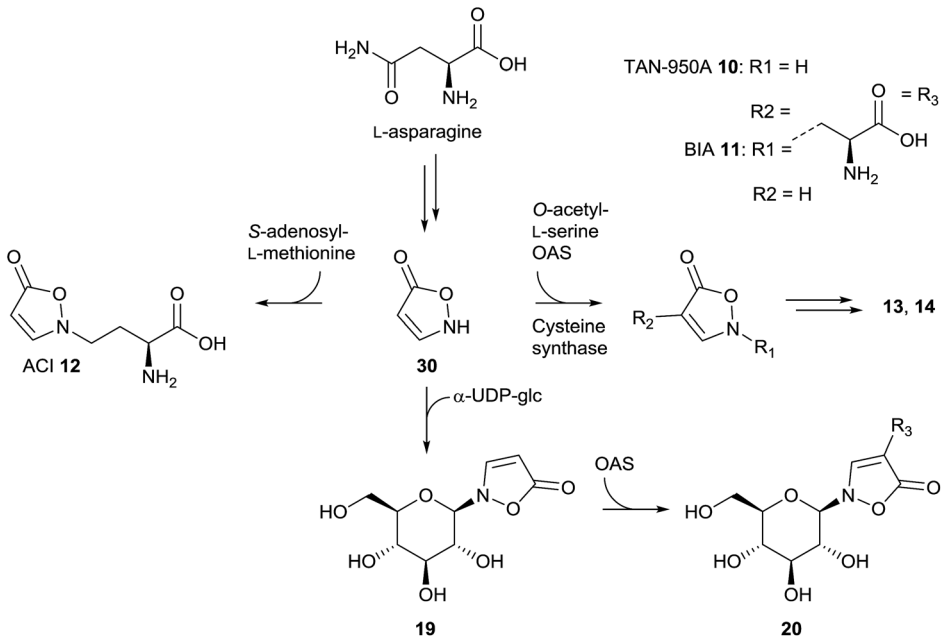

Fig. 8 Proposed biosynthetic pathways for the biosyntheses of isoxazolin-5-one derivatives 10 to 14, 19, 20 and 30 in different plant species, reconstructed from the cited literature.

hydroxypropionitrile **38** as well as 3-bromopropanoic acid **39** (Fig. 9c).[5,37,41] The yield of the last step of the latter method was further improved by using $AgNO_2$ as a source for the nitro group instead of $NaNO_2$.[116] Previous attempts using 3-iodopropanoic acid and silver nitrite in water yielded only 14% of the product.[117]

Furthermore, the synthesis of 3-NPA **1** using β-propiolactone **40** and sodium nitrite in one step and giving a moderate yield was described (Fig. 9d).[118]

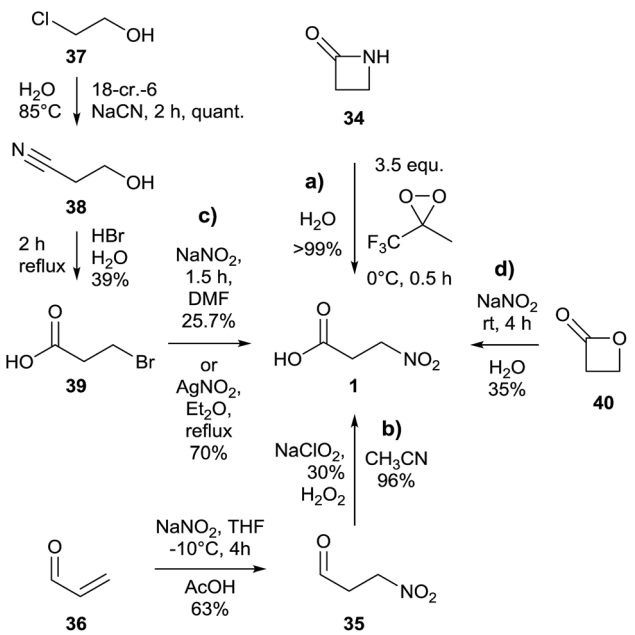

Fig. 9 Synthetic procedures for the synthesis of 3-nitropropanoic acid.

4.2. Syntheses of naturally occurring isoxazolin-5-one derivatives

Syntheses of *N*-substituted 3-isoxazolin-5-one derivatives were achieved using the sodium salt of the heterocycle **30**.[59,72,119,120] The latter compound was synthesized starting from ethyl propiolate **41** *via* ethyl 3-(hydroxyimino)propanoate **42** (Fig. 10a).[80]

Many attempts with alkyl bromides and chlorides failed or gave only low yields, as in the case of compound **19**, which was synthesized *via* 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **43** (Fig. 10b).[59,119] This observation is in part due to the low selectivity of these reactions, which leads to the formation of 3-, 4- as well as *O*-substituted derivatives of 3-isoxazolin-5-one.[119] A further attempt where *O*-acetylserine reacted with isoxazolin-5-one under catalysis by pyridoxal phosphate provided BIA **11** in a very low yield of 0.15%.[121]

Thus, an alternative method for the synthesis of 3,4-unsaturated 3-isoxazolin-5-one derivatives was developed by Baldwin and coworkers, based on a 5-*endo*-dig reaction.[122,123] The construction of the heterocycle proceeds *via* *O*-acylation of an (*N*-boc-protected) *N*-hydroxy derivative with propynoic acid **45** to form compound **44**, followed by an attack of the triple bond by the amino functional group, released upon formic acid-catalyzed deprotection (Fig. 10c).[123] Upon application of this 5-*endo*-dig strategy, the naturally occurring compounds BIA **11** as well as the glucoside **19** could be synthesized (Fig. 10b and c).[58,122–124] Compound **19** can be obtained directly from D-glucose in two steps and one pot.[124] Upon transesterification using *Candida antarctica* lipase B (CALB) as well as an trichloro ester **46**, compound **4** can be synthesized in moderate yields.[58] This provides rapid access to $^{13}C_6$-labeled **4** from [$^{13}C_6$]-**19**, which is easily accessible from commercial [$^{13}C_6$]-D-glucose. The yields of the obtained products are sufficient to provide the required

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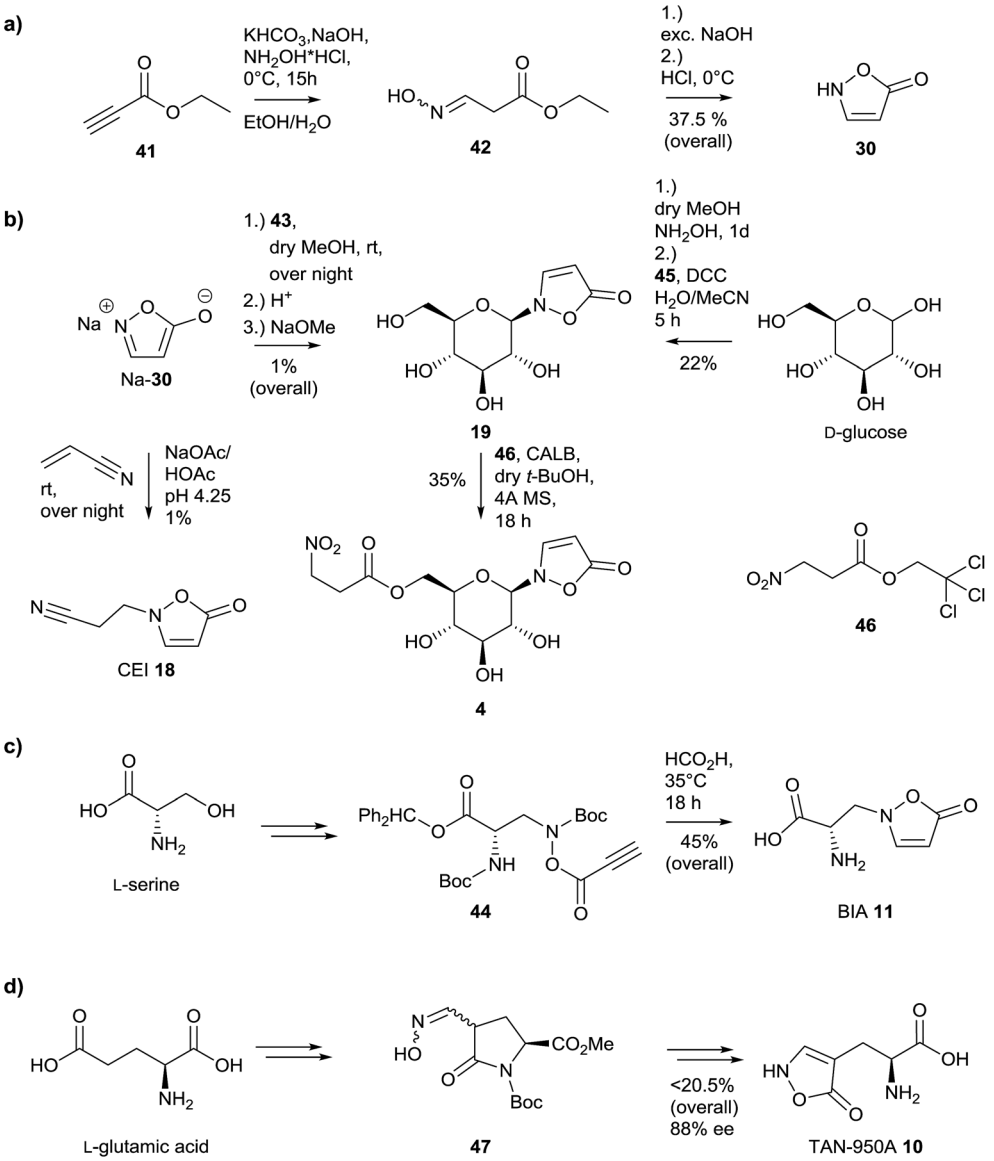

Fig. 10 Synthetic strategies to provide naturally occurring 3-isoxazolin-5-one derivatives **4**, **10**, **11**, **18**, **19** and **30**, adapted from the literature.

amounts of authentic standards for qualitative and quantitative analyses.[5,41,123] Using these standards, aspects of the biosynthesis of these natural products can be studied using synthetic isotopically labeled intermediates.[5,104,123] Furthermore, quantifications can be simplified and are more reproducible if samples are spiked with defined amounts of stable-isotope-labeled internal standards (SIL-IS).[5,125]

A further synthesis was described for the construction of compound **10**, starting from L-glutamic acid (Fig. 10d).[76,126,127] The synthesis of TAN-950A proceeds over six steps from L-glutamic acid *via* oxime **47** in moderate yield and with a loss of 12% of the initial optical purity. The synthetic protocol has also been applied to D-glutamic acid resulting in an enantiomeric excess of 72%.

In the case of benzisoxazolinones, synthetic models for parnafungins were described.[128,129] For further studies on the synthesis and properties of isoxazolones as well as -5-oles and related compounds see Beccalli[130] and Sørensen[131] *et al.*

4.3. Properties of isoxazolin-5-one derivatives

Aqueous solutions of unsubstituted 3-isoxazolin-5-one **30** show rapid degradation, forming cyanoacetic acid as a main product.[80] Under alkaline pH, compound **30** shows increased stability.[80] In contrast, *N*-substituted 3-isoxazolin-5-one derivatives show rapid degradation in alkaline media upon hydrolysis and are stable under acidic conditions.[59,124,132] The stability at low pH that was observed for many 2-substituted 3-isoxazolin-5-one derivatives, especially in the corresponding *N*-glucosides,[124,132] was unexpected, due to the general instability of glycosides under acidic conditions. The stability of glucoside **19** persists even in the presence of β-glucosidase from almonds.[124] In different solvents, the content of all three possible tautomers

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of compound **30** changes.[80] Under alkaline conditions, the anionic form of the corresponding isoxazol-5-ol predominates, while in chloroform the 2-isoxazolin-5-one isomer is mainly observed.[80] Under acidic conditions, the content of the 3-isoxazolin-5-one increases to 55%.[80]

Upon irradiation at wavelengths around the absorption maximum (263 ± 3 nm) of 2-substituted 3-isoxazolin-5-one derivatives, the heterocycle decomposes efficiently, forming a range of different products such as corresponding amines as well as *N*-acetyl amines and CO_2.[133] The quantum yields of these photohydrolysis reactions were determined to be above 0.3 in naturally occurring 3-isoxazolin-5-one derivatives.[59,124,133]

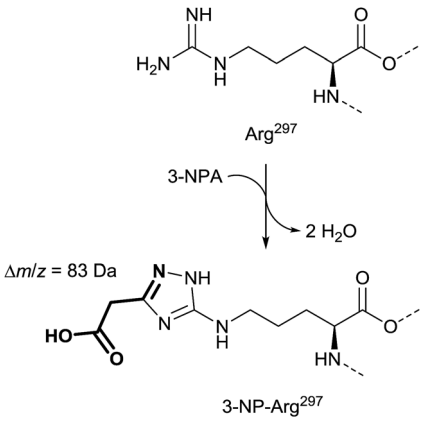

Fig. 12 Mass spectra and X-ray supported suggestion for the structure of the side-chain of Arg^{297} in 3-NPA-treated enzyme (bottom), compared with the Arg^{297} side-chain in the untreated complex II (top), adapted from Huang *et al.*[136]

5. Toxicology

5.1. Toxic mechanisms of 3-nitropropanoic acid

3-Nitropropanoic acid **1** is a mitochondrial toxin due to its ability to irreversibly inhibit the generally occurring enzyme succinate dehydrogenase.[134] This property is due to the isoelectronic character of 3-NPA compared to succinic acid (Fig. 11), which is the natural substrate of mitochondrial complex II.

Thus 3-NPA **1** is able to bind effectively to the catalytic center of succinate dehydrogenase.[135] Several species deriving from 3-NPA that might deactivate succinate dehydrogenase have been suggested, *e.g.* 3-nitroacrylate,[136] which could be formed due to oxidation of 3-NPA catalyzed by complex II, unaltered 3-NPA,[135] the (dianionic) 3-nitronate isomer (P3N)[35] or even its carbanion.[134]

X-ray as well as mass spectrometry analyses demonstrated that a covalent adduct of 3-NPA and the enzyme is formed, which supports the observation of succinate dehydrogenase as an irreversible inhibition mentioned.[136] Furthermore, X-ray diffraction provides evidence for the formation of an unusual cyclic adduct between the guanidino group of the side-chain of Arg^{297} and 3-NPA within the catalytic center. This product is reconstructed from the original publication to bear most likely a 1,2,4-triazole moiety (Fig. 12).[136]

The suggested structure is supported by MS analyses of tryptic peptides before and after treatment of complex II with 3-NPA, indicating a mass shift of 83 Da in the digestive fragments after addition of 3-NPA to solutions of complex II. In addition, the tryptic peptides show losses of 44 Da, which indicates decarboxylation, and thus the involvement of the nitro group instead of the carboxylic acid of 3-NPA in the formation of the covalent adduct.[136] Alternative covalent addition products, *e.g.* with the contribution of the flavin cofactor[35] or other functional groups in the enzyme,[137] *e.g.* mercapto groups, were not indicated by these[136] data.

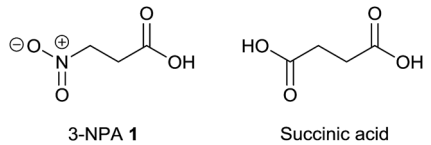

Fig. 11 Structures of 3-NPA and succinic acid.

Whether the formation of this covalent addition product requires the complex II catalyzed oxidation of 3-NPA to form 3-nitroacrylate within the catalytic center, prior to inactivation, is still under investigation.[136] The former experiments indicated a rapid inactivation of complex II upon application of 3-nitroacrylate within 15 s, but four to five equivalents of the inhibitor were required in the case of 3-nitroacrylate, while in the case of 3-NPA one equivalent was sufficient for inactivation.[138]

A previous study based on the crystal structure of complex II binding 3-NPA shows a non-covalent complex between substrate and enzyme.[135] These findings were mainly supported by X-ray data of lower resolution and they do not explain the observed irreversible inhibition. Thus, these structures might show a pre-inactivation complex between the enzyme and 3-NPA.

Although the observed irreversible inhibition of complex II leads to mitochondrial energy impairment due to interference with the tricarboxylic acid cycle (Krebs cycle), the lethal dose in mice and rats indicates medium acute toxicity after oral administration, with LD_{50} values between 60 and 120 mg kg^{-1} body weight.[139] Nevertheless, chronic 3-NPA administration causes neurodegeneration.[140,141] The effects caused by 3-NPA are similar to the symptoms of Huntington's disease,[142,143] and thus compound **1** was used as a tool to study this illness, as it induced the symptoms in animal models.[4,144,145] Typical symptoms of Huntington's disease include, in particular, dysfunctions of movement such as dystonia, chorea, and hypokinesia.[143] 3-NPA intake was shown to poison cattle that fed on food that contained this toxin.[142] Significant economic damage occurs due to poisoning by the ingestion of plants or microorganisms, *e.g.* from moldy sugarcane containing *Arthrinium* spp.[15]

Due to the obvious general toxicity of 3-NPA, the toxin was also characterized in terms of some antitumor toxicity using human cell lines.[146]

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In addition to the toxicity against mammalian organisms, 3-NPA showed toxicity against many other cell types, *e.g.* against *Mycobacterium tuberculosis* H37Ra[147] as well as H37Rv,[10] due to the general toxicological mechanism of compound **1**. Different minimum inhibition concentrations (MIC) have been published, in the range of 3.3 μM and 50 μg mL^{-1} (420 μM) for H37Ra[10,147] and 12.5 μg mL^{-1} (105 μM) for H37Rv.[10]

For a more comprehensive overview of toxicological parameters and corresponding experiments see Burdock *et al.*[139] as well as Madhusudan *et al.*[142]

5.2. Detoxification of 3-NPA

Since toxic 3-nitropropanoic acid **1** occurs in many plants and leaf beetles as well as in microbiota, different detoxification mechanisms have evolved in various organisms that are exposed to significant doses of 3-NPA. In insect generalists, *e.g.* *Melanoplus* species[148] or *Spodoptera littoralis*,[149] which feed on leguminous plants among many others, it was shown that 3-NPA detoxification is carried out by the formation of amino acid amides (Fig. 13).

After the formation of these amino acid conjugates, *e.g.* derived from glycine **48**, alanine **49** and glutamic acid **50**, the amides are excreted *via* the malphigian tubules over the hind gut of the insects.[149]

In (leguminous) plants, the ability to detoxify 3-NPA **1** *via* oxidation probably evolved to protect sensitive tissue from intoxication.[16,150] This oxidation is catalyzed by 3-NPA oxidase, which delivers malonate semialdehyde **33**, nitrite, nitrate and hydrogen peroxide (Fig. 13).[16,150] The activity of this enzyme in various leguminous plants correlates with the amount of 3-NPA that is produced by the organism.[16]

Similar reactions have been observed in *P. atrovenetum*, where a flavoprotein was identified as oxidizing 3-NPA (or its homologue propionate-3-nitronate) to produce compound **33** and a number of further compounds, *e.g.* superoxide.[151] When *E. coli* cells were genetically modified to produce this oxidase, the bacteria were shown to be protected from 3-NPA mediated toxicity.[152]

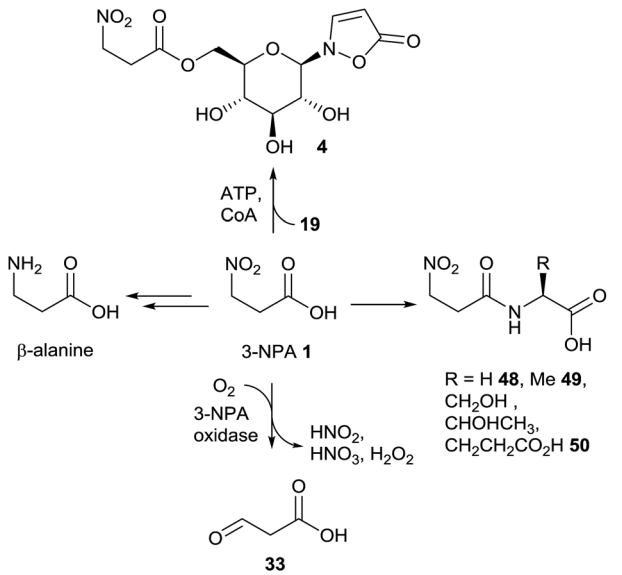

Fig. 13 Detoxification pathways of 3-NPA in different organisms.

Just as the consumption of propionate-3-nitronate[151] was indicated by a decrease of oxygen concentration upon the addition of alkaline 3-NPA solution to the medium, the fungal protein was named as propionate-3-nitronate oxidase.[151] In these studies, buffered solutions with pH values of around 8 were applied, yielding higher contents of the nitronate species, but no optimum pH range of the enzyme was determined.[151] In contrast, the plant-produced 3-NPA oxidase showed maximum activity within a slightly acidic reaction medium (pH 5).[150] Thus, in plants the occurrence of a nitronate dianion species, prior to oxidative cleavage of 3-NPA, is unlikely to be required.

It has been shown that mixed cultures of microbiota from the ruminal gut can metabolize 3-NPA to form β-alanine (Fig. 13).[153] This pathway is of interest in so far as it explains the adaptation of ruminants to this toxin.[154,155]

In leaf beetles, the prevention from self-intoxication with 3-NPA **1** is realized by the formation of the ester compound **4** (Fig. 13).[41] After the injection of [1-^{13}C,^{15}N]-3-nitropropanoic acid into larval hemolymph, isotopically labeled product **4** was detected, showing a mass shift of $\Delta m/z = 2$. This detoxification hypothesis is supported by sufficient concentrations of the glucoside precursor, compound **19**, exceeding the amount of the corresponding nitro-ester **4** in early larval stages.[5,41] The formation of the ester compound **4** equals the last step in the biosynthesis of this compounds in leaf beetles (Chrysomelina), as shown in the biosynthesis section in this article.

5.3. Toxicology of isoxazolin-5-one derivatives

Diverse non-natural isoxazolin-5-ones, isoxazol-5-oles and related compounds show promising biological activity, and thus are used as drugs for diverse targets.[156–158] In naturally occurring isoxazolinones, various experiments have been carried out to explore the corresponding ecological relevance as well the application potential of these compounds. Since it was observed that the consumption of plants belonging to the genus *Lathyrus*, *e.g.* *Lathyrus sativus* (grass pea), causes the neuronal disease neurolathyrism,[159] many studies concerning biological functions of natural isoxazolinones have focused on the involvement of such compounds in corresponding neuropathologies.

In vitro investigations concerning the non-proteinogenic amino acid BIA **11** and its homologue ACI **12** showed that compound **11** has some excitotoxic potential.[160] The excitotoxic potential of **11** was lower or in some cells comparable to the values of the well characterized neurotoxin β-*N*-oxalyl-L-α,β-diaminopropanoic acid (β-ODAP), which occurs together with BIA **11** in leguminous plants. The application of 6-cyano-7-nitroquinoxaline-2,3-dione indicated that the toxicity of **11** was mediated by non-*N*-methyl-D-aspartate type receptors.[160] Compound **12** showed no significant excitotoxic effects in these experiments, but it was observed previously that neurolathyrism in young chicks was caused by this substance.[161] Since these compounds were readily available from natural sources, further toxicological aspects have been investigated, *e.g.* antibiotic

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activity. In the case of BIA **11**, antifungal activity, including mycelial as well as cell growth inhibition, has been observed in several strains of yeast species, without showing any antibacterial activity.[162] A minimum inhibitory concentration (MIC) of 3 μM for BIA **11** was determined for *Saccharomyces cerevisiae* in minimal medium.[163] Thus, compound **11** may play a significant role as a plant-protecting agent by influencing the rhizosphere, especially during early developmental stages in young legumes.[162]

Compound **10** (TAN-950A), which is a structural analogue of BIA **11**, showed inhibitory effects on an L-glutamate/L-aspartate transporter (GLAST) and is significantly expressed in glial cells.[164,165] In these experiments, no effects were observed for the corresponding glucoside **20**.[164] Furthermore and analogous to BIA **11**, TAN-950A **10** was identified as an antifungal antibiotic agent, showing activity against some *Candida* strains as well as *Saccharomyces cerevisiae* with minimum inhibitory concentrations of 0.78 to 3.13 μg mL^{-1}.[166] It was demonstrated that activity persisted in response to the application to systemic *Candida albicans* TA infections in mice.[166]

In the case of the naturally occurring benzisoxazolin-5-one derivatives, promising bioactivities have been observed. It was demonstrated by using the *Candida albicans* Fitness Test that the parnafungins are antifungal agents,[77,167,168] inhibiting mRNA polyadenylation (for the structure of parnafungin B1 **21** see Fig. 4). It was observed by affinity selection/mass spectrometry that the "straight" isomer, assigned as parnafungin A, binds with higher affinity to polyadenosine polymerase (PAP) compared to the "bent" structures of other parnafungins.[169]

The marine sponge secondary metabolite bromobenzisoxazolone barettin **22** was identified as an antifouling agent, as it inhibits the settlement (EC_{50} = 15 nM) of barnacle larvae (*Balanus improvisus*).[9] The efficiency of this inhibition is about 60 times more potent than that of isolated structural analogues, which lack the benzisoxazolinone moiety.[9]

The *N*-hydroxybenzisoxazolinone **23** showed antibacterial activity against *V. anguillarum*, *V. harveyi* VIB 286, *A. hydrophila* and *S. aureus* in TLC bioautography overlay assays.[8]

Since antifungal properties have been observed in a number of isoxazolin-5-one derivatives, glucoside **19** was tested against various cell lines of human and microbial cells. For this purpose, solutions of glucoside **19** (0.1 to 1 mg mL^{-1}) were added to cell lines of phyto- and entomopathogens as well as to human pathogens.[170] None of those tests resulted in any visible inhibition of growth of the cells or the mycelium of the treated cell lines. Analogously, neither increased mortality nor repulsion of *Myrmica rubra* (Formicidae) individuals feeding on 10^{-2} M solutions of compound **19** was observed.[44] These observations indicate that some of the natural isoxazolinone derivatives may not cause (neuro-)toxicity. In the case of compound **19**, a major ecological benefit for its producers may arise from derivatization with a 3-nitropropanoyl residue, which allows high amounts of this toxin to be stored, as shown in the case of leaf beetles.[41] This hypothesis is further supported by a significant deterrent effect of compound **4** against *M. rubra*,[44] which shows that storage of 3-NPA in the form of the corresponding ester in the leaf beetle hemolymph is non-toxic for the organism.

Although this hypothesis is plausible, many plants produce simple glucose esters of 3-NPA, without the presence of the isoxazolin-5-one moiety, as shown in Section 2. Furthermore, high amounts of the 3-NPA free glucoside **19** as well as many other isoxazolin-5-one derivatives of ambiguous biological function occur in various species of different kingdoms. These observations may indicate the as-yet undiscovered ecological importance of many natural isoxazolin-5-one derivatives, beyond a role within neurotoxicity or antibiotic activity.

6. Conclusions and perspectives

Natural compounds derived from 3-NPA and isoxazolin-5-one occur throughout four kingdoms of biology and show considerable structural diversity. In several species of Chrysomelina leaf beetles and leguminous plants of the genus *Astragalus*, both structural motifs occur at the same time an in the same molecule. In most cases, it is still unclear whether such a relationship occurs within 3-NPA and/or isoxazolin-5-one producers. Furthermore, whether 3-NPA is produced as a pure compound, or as a derivative, *e.g.* an ester, is not yet apparent.

Biochemical fundamental research about the neurodegenerative toxicity of 3-NPA has been carried out, and results show that this nitro acid irreversibly inhibits the mitochondrial respiration *via* formation of a cyclic addition product with succinate dehydrogenase. Whether the unaltered compound 3-NPA is involved in the final deactivation, or its oxidized form 3-nitroacrylate, or the corresponding dianionic tautomers propionate-3-nitronate and the 3-deprotonated carbanion is under investigation.

These toxicological properties mainly contribute to the ecological function of 3-NPA as a general poison, one that is produced in significant amounts by plants and beetles as well as fungi. Nevertheless, it seems that there are no studies specifically aimed at investigating the evolution of the interactions between the organisms producing 3-NPA and isoxazolin-5-one derivatives and their predators, as have been carried out for producers of cardenolides,[171] iridoids[172] and nicotine.[173] Evidence for a defensive function in the case of leaf beetles is indirect, deduced from the secretion of compounds in well-established defensive glands; further evidence is found in defensive toxins that are produced by species of other taxa, *e.g.* cardenolides[174] and triterpene saponins,[51] which release the secretion in response to disturbance, and from the obvious general toxicity of 3-NPA.

As widespread occurrence of 3-NPA and its derivatives shows, several fundamentally different detoxification mechanisms have evolved in various species of different kingdoms. The detoxification pathways include ester or amide formation with sugars and with amino acids. These reactions increase the storage potential of 3-NPA and protect the organism that produces it. Furthermore, it has been suggested that 3-NPA can be reduced to β-alanine by certain microorganisms or cleaved upon oxidation to form malonate semialdehyde, hydrogen

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peroxide, nitrate and nitrite, as well as other products, as shown in microbiota and (leguminous) plants.

In the case of isoxazolin-5-one derivatives, toxicological investigations were carried out to analyze their neurotoxicity as well as antibiotic and antifouling properties. Some promising results were found, although the biological function of many other isoxazolinone natural products, *e.g.* compound **19**, still seems obscure.

Biosynthetic pathways have been studied in legumes and fungi, as well as leaf beetles. In fungi, MS, NMR and radioactivity experiments including degradation procedures to localize the incorporated label were conducted, resulting in the discovery of aspartate as the ultimate precursor. This concept was transferred to leaf beetles, resulting in positive radioactive labeling after aspartate feeding. MS investigations later showed that in Chrysomelina larvae, aspartate is incorporated through random re-assembly rather than direct incorporation. Instead of a metabolic route with aspartic acid as the ultimate precursor, a pathway starting from L-valine *via* propanoyl-CoA and β-alanine leads to the direct formation of isoxazolin-5-one and 3-NPA derived natural compounds. In the leguminous plant *I. spicata*, an alternative pathway for the biosynthesis of 3-NPA was suggested on the basis of radioactive labeling experiments. These studies resulted in the positive incorporation of malonate, while aspartate failed to be incorporated into 3-NPA. Whether alternative pathways, *e.g.* from valine and propanoyl-CoA, lead to a more direct production of 3-NPA in legumes has not yet been investigated. The formation of isoxazolin-5-one derivatives in plants has been studied independently from the biosynthesis of 3-NPA, resulting in the positive incorporation of isoxazoline-5-one as a general precursor for corresponding natural products. This compound can be transferred to various acceptors in plants, *e.g. S*-adenosyl-L-methionine, *O*-acetyl-L-serine or α-UDP glucose, leading to the biosynthesis of various natural compounds. Nevertheless, many questions regarding the biosynthesis of isoxazolin-5-one derivatives in plants remain unanswered, especially with respect to the exploration of corresponding biosynthetic intermediates and ultimate precursors. Whether a relationship between 3-NPA and isoxazolin-5-one production occurs in plants, as it does in leaf beetles,[5] is unknown. Furthermore, it is of interest whether the biosyntheses are due to autogenous *de novo* production or to the involvement of (*endo*-)symbionts, or to both, especially in animals and plants producing 3-nitropropanoic acid or derived compounds.

The ecological importance of isoxazolin-5-one and of 3-NPA derived natural compounds for their producers can be better understood once the details of their biosynthetic pathways have been investigated; these details include related genes and enzymes, especially those supported by knock-out experiments which provide genetic information and enable studies of chemo-ecological aspects. Genetic information can also improve the health effects and safety of important food plants, *e.g. L. sativus* and *P. sativum*, by making use of tailored genetic engineering. Gene clusters showing *in vitro* activity for the production of 3-NPA from aspartate have recently been described in some *Streptomycetes* strains.[93]

In order to unravel important aspects of occurrence, amounts, biological properties as well as the biosyntheses, the chemical synthesis and subsequent qualitative and quantitative analyses of these natural products and their putative biosynthetic intermediates can be a useful tool. Some of the naturally occurring isoxazolinones have already been synthesized, but due to multiple synthetic steps, the tailored incorporation of isotope labels remains challenging. Nevertheless, some attempts using synthetic isotope labeled isoxazolinone glucosides have led to the improvement of quantitative analyses (SIL-IS).[5,125]

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