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METABOLIC ROUTES OF β-(ISOXAZOLIN-5-ON-2-YL)-L-ALANINE (BIA), THE PRECURSOR OF THE NEUROTOXIN ODAP (β-*N*-OXALYL-L-α,β,-DIAMINOPROPIONIC ACID), IN DIFFERENT LEGUME SEEDLINGS

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Key Word Index—*Lathyrus sativus*; *L. odoratus*; *Lens culinaris*; *Pisum sativum*; *Leguminosae*; isoxazolinone derivatives; β-*N*-oxalyl-α,β-diaminopropionic acid; neurotoxin; incorporation; secondary metabolism.

Abstract—BIA, β-(isoxazolin-5-on-2-yl)-alanine, is formed in high concentrations in the seedling stage of many plants belonging to the tribe *Vicieae*. The metabolism of this compound in different legume seedlings has been studied by radioisotope feeding. In *Lathyrus sativus*, BIA is metabolised to the neurotoxin ODAP (β-*N*-oxalyl-α,β-diaminopropionic acid) and the γ-glutamyl derivative of BIA. In *L. odoratus* seedlings BIA is metabolised to the decarboxylation product of the glutamyl derivative, and the glutamyl derivative is proposed as an intermediate. In *Pisum sativum* seedlings, the main part of BIA is not further metabolised. While in *Lens culinaris* seedlings, besides the formation of the glutamyl derivative, another four unknown amino acids were found. Approaches for the detoxification of *Lathyrus sativus* by genetic transformation are suggested.

INTRODUCTION

Legumes are a rich source of secondary metabolites, especially unusual non-protein amino acids, some of which are toxic to men and domestic animals. In the seedling stage of some *Vicieae* plants a group of heterocyclic isoxazolinone derivatives is formed in high concentrations [1, 2]. Among them β-(isoxazolin-5-on-2-yl)-L-alanine or BIA is most common and was first isolated from the garden pea (*Pisum sativum*) seedlings. Later it was found to be present in most seedlings of the *Vicieae* tribe including food and feed plants such as all species of the genera *Lens* and *Pisum* examined, most *Lathyrus* species examined and some *Vicia* species. Some *Lathyrus* species are exceptionally rich sources for isoxazolinone compounds; ten such compounds with different side chain attached to the nitrogen of the five-membered ring have been identified, some of them toxic (Scheme 1). Their distribution in several legume seedlings is shown in Table 1. Like most other secondary metabolites, the physiological function and the metabolism of this group of isoxazolinone compounds in plants are not very well understood.

Neurolathyrism is a human upper motor neurone disease which affects the lower limbs of the patients after prolonged consumption of *Lathyrus sativus* seeds in some Asian and African countries. This disease occurs mainly during the drought and famine years [4]. A small non-protein amino acid, β-ODAP (β-*N*-oxalyl-L-α,β-diaminopropionic acid) with neuroexcitotoxic properties was proposed as the cause of this disease [5]. We recently demonstrated that BIA is the biosynthetic precursor of the neurotoxin β-ODAP in different stages of *Lathyrus sativus* plants: in germinating seeds [6], in ripening pods [7] and also in callus tissues [8, 9]. DAPRO (α,β-diaminopropionic acid) was proposed as the short-lived intermediate between BIA and ODAP.

Because BIA is the precursor of the neurotoxin ODAP, we also examined the possible neuroexcitotoxicity of this compound in neurone cells as compared to β-ODAP under the same condition. It was found that BIA at 0.5–2.0 mM produced a concentration-dependent neurodegeneration in mouse cortical explants and this action was mediated by non-NMDA type receptors [10]. But the excitotoxic potential of BIA is less than β-ODAP because in some cells BIA in 2 mM concentration produced currents which were similar in amplitude to those activated by β-

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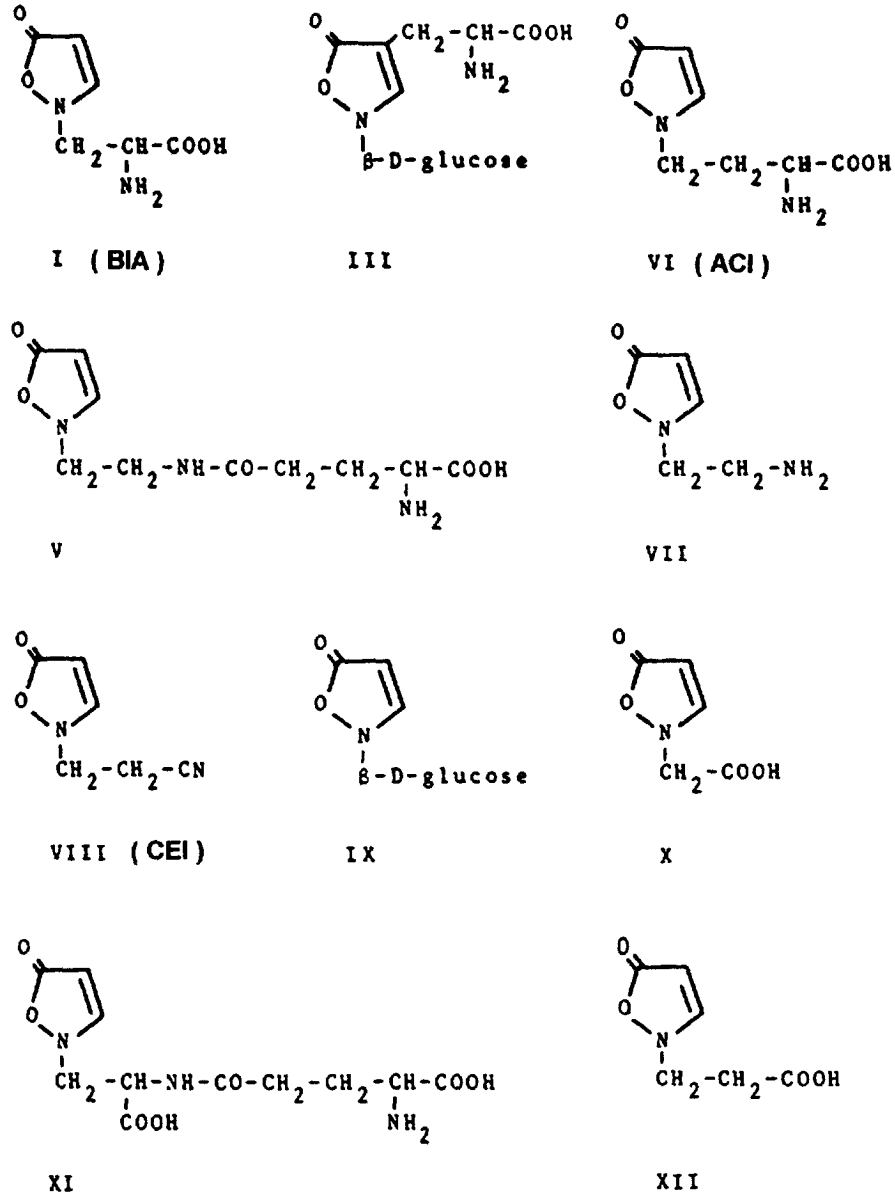


Scheme 1. Natural occurring isoxazolin-5-one derivatives isolated from some *Vicieae species*. The roman numerals used to identify the compounds are those that have been used by us in previous studies.

Table 1. Distribution of isoxazolinone derivatives and the neurotoxin ODAP (*β*-*N*-oxalyl-α,*β*-diamino-propionic acid) in legume seedlings

	I	III	V	VI	VII	VIII	IX	X	XI	XII	ODAP
Pisum sativum	+	+	—	—	—	—	—	—	—	—	—
Lathyrus odoratus	+	+	+	+	+	+	+	+	—	+*	—
Lathyrus sativus	+	+	—	+	—	+	—	—	+	—	+
Lens culinaris	+	+	—	—	—	—	—	—	+	—	—

* Only found in immature seeds [3].
I: BIA; VI: ACI; VIII: CEI.

ODAP in 50 μM concentration. In another study with rat brain NMDA receptor binding assay, BIA was inactive while β-ODAP showed slightly inhibitory activity (IC_{50}: 47 μM) [11]. Recent work with cloned mouse glutamate receptors expressed in *Xenopus* oocytes has confirmed the previous results that BIA did not show any effect on the NMDA binding site while a weak agonistic action on AMPA receptors at 0.5 mM concentration was observed [12].

Besides its weak excitotoxic activity towards mammalian cells, BIA was suggested to play an ecological role as an allelochemical in the plants producing it. In the young seedlings of *Pisum sativum* and *Lathyrus odoratus*, BIA and other isoxazolinone compounds are actively exuded from the roots in higher concentrations than other protein amino acids [13]. BIA was shown as a potent growth inhibitor towards yeast, phytophathogenic fungi, unicellular green algae and also some non-legume higher plants [14, 15]. However, no significant inhibition of either Gram-positive or Gram-negative bacteria was observed by BIA. *Rhizobium* spp. were able to tolerate high concentration of BIA up to 2.9 mM. These results suggest an ecological role of BIA as a protectant for the plant in the seedling stage against micro-organisms except the beneficial symbiotic *Rhizobium* spp.

Considering the biological and ecological importance of BIA, and the observation that only in some *Lathyrus* species it is the precursor of a neurotoxin, we have studied the metabolism of this compound in different legume seedlings.

RESULTS AND DISCUSSION

Since BIA is the major isoxazolinone compound in the young seedlings of *L. sativus*, we studied the metabolism of this compound in the germinating seed by feeding ^{14}C-labelled BIA soln to the imbibing seeds. The uptake of radioactivity was about 99%. After the seeds had grown in vermiculite for 2 days, the embryo axes and the cotyledon part were harvested and a 70% ethanol extract was prepared and analysed by high voltage paper electrophoresis respectively. The two major radioactive peaks were identified as ODAP and BIA in both the embryo and the cotyledon part. In the cotyledon part, two additional radioactive bands were present containing some 15% of the total radioactivity of the 70% EtOH extract. The percentage radioactivity in the 70% EtOH extracts is shown in Table 2. The eluate of the UV band of BIA contained another isoxazolinone compound designated as compound XI (γ-glutamyl-BIA).* When the eluate of this UV band was applied on 2D TLC followed by autoradiography, two UV spots showing distinct radioactivity were identified as BIA and compound XI. The spot of BIA in the embryo material showed higher radioactivity than in cotyledons. The specific radioactivity of BIA (0.62 μCi/mmol) and XI (0.49 μCi/mmol) purified from the embryo part, was very similar, indicating that these two compounds are metabolically very closely related. These results suggest that in *L sativus* seedlings BIA is metabolised by two routes simultaneously to form the neurotoxin ODAP and also the γ-glutamyl derivative of BIA (compound XI) in both embryo and cotyledon. While other isoxazolinone compounds ACI (2-(3-amino-3-carboxypropyl)-isoxazolin-5-one) and CEI (2-cyanoethyl-isoxazolin-5-one) present in the seedlings of *L. sativus* were not labelled.

Table 2. Percentage of total radioactivities of embryo and cotyledon in 70% EtOH extracts of 2-day-old seedlings of *L. sativus* after [^{14}C]BIA (total act. 98,000 cpm) was fed to the imbibing seeds (10 seeds)

	Embryo	Cotyledon
Band ODAP	14.8%	10.4%
Band BIA (+XI)	40.6%	23.3%

In young *L. odoratus* seedlings, BIA and ACI are quantitatively the most important isoxazolinone compounds. In 10-day-old seedlings, ACI makes up 3.5% of the seedling dry weight, followed by BIA (1.9%) and compound V: γ-glutamyl-2-aminoethyl-isoxazolin-5-one (1.2%) [16]. ACI is the higher homologue of BIA with one more carbon in the side chain. However, the biosynthesis of these two compounds in plants was shown to be quite different. BIA is formed by cysteine synthase with the free isoxazolin-5-one ring and *O*-acetyl-L-serine as substrates [17]. In seedlings of both *Lathyrus sativus* and *Pisum sativum*, cysteine synthase isoenzyme B showed a slightly higher activity than isoenzyme A in forming BIA. With purified enzymes *in vitro*, the activity of cysteine synthase enzymes to form BIA is very low compared to that of cysteine formation: 0.06–0.1% in *P. sativum* and 0.07–0.08% in *L. sativus*. *O*-Acetyl-L-homoserine, the higher homologue of *O*-acetyl-serine, could not serve as the donor of the side chain for ACI, the higher homologue of BIA. The biosynthesis of ACI is from the free isoxazolin-5-one ring with S-adenosyl-methionine (SAM) as the donor of the 3-amino-3-carboxypropyl side chain in the enzyme preparation from *L. odoratus* seedlings [18]. Compound V which is structurally very similar to compound XI except for the carboxylic acid group of the alanyl-moiety, was until now only found in the seedling of *L. odoratus* and was not detected in any other legume seedlings examined.

The incorporation of [^{14}C]BIA in germinating seeds of *L. odoratus* showed a high percentage uptake of the radioactivity, up to 96%. Extraction of 3-day-old seedlings (because *L. odoratus* grows slower than

* The roman numerals used to identify the compounds are those that have been used by us in previous studies.

Table 3. Percentage of total radioactivities of embryo and cotyledon in 70% EtOH extracts of 3-day-old seedlings of *L. odoratus* after [^{14}C]BIA (total act. 40,058 cpm) was fed to the imbibing seeds (5 seeds)

	Embryo	Cotyledon
Band BIA	7.5%	11.4%
Band ACI (+V)	18.3%	29.4%

L. sativus) and analysis by paper electrophoresis, showed the major radioactive peaks localised in the band of BIA and the band of ACI. The percentage of total radioactivities in 70% EtOH extracts is shown in Table 3. The ACI band contains also compound V. ACI and V could not be separated by high voltage paper electrophoresis under the conditions used but they could be well separated by 2D TLC as above. Autoradiography of the 2D TLC showed that the spot of compound V was labelled but not the spot of ACI which was present in much higher concentration. Therefore, in *L. odoratus* seedlings BIA is mainly metabolised to compound V.

Since structurally compound V is the decarboxylation product of compound XI and compound XI was not detected in *L. odoratus* seedlings, it is possible that compound XI is a short-lived intermediate between BIA and compound V in *L. odoratus* seedlings similar to the situation in *L. sativus* seedlings, where DAPRO (α,β-L-diaminopropionic acid) is the intermediate between BIA and ODAP. To confirm this hypothesis we isolated ^{14}C-labelled compound XI from 2-day old *L. sativus* seedlings which incorporated [U-^{14}C]serine at the imbibing seed stage. Unlike the easy uptake of BIA by the seeds, a preliminary test showed that compound XI is not readily taken up by the imbibing seeds. Only when compound XI was dissolved in 0.01 M KH_2PO_4 buffer, pH 7.0, did the uptake of the radioactivity reach 72.8%. ^{14}C-labelled compound XI was taken up into the imbibing *L. odoratus* seeds, which then were germinated for 3 days. In the 70% EtOH extracts of the embryo and the cotyledons, the major radioactive peaks were the band of compound XI and the band of ACI (+V) (Table 4). The band of ACI (+V) was further analysed by 2D TLC, followed by autoradiography. The results showed that compound V was indeed labelled while ACI was not labelled, neither in the embryo nor in the cotyledon extract. To ensure that compound XI as such was taken up and not after breakdown to BIA, the presence of unchanged compound XI was confirmed by the presence of radioactive XI in the *L. odoratus* seedling extracts and also in the media after the seeds were incubated.

Table 4. Percentage of total radioactivities of embryo and cotyledon in 70% EtOH extracts of 3-day-old seedlings of *L. odoratus* after [^{14}C]XI (total act. 190,000 cpm) was fed to the imbibing seeds (12 seeds)

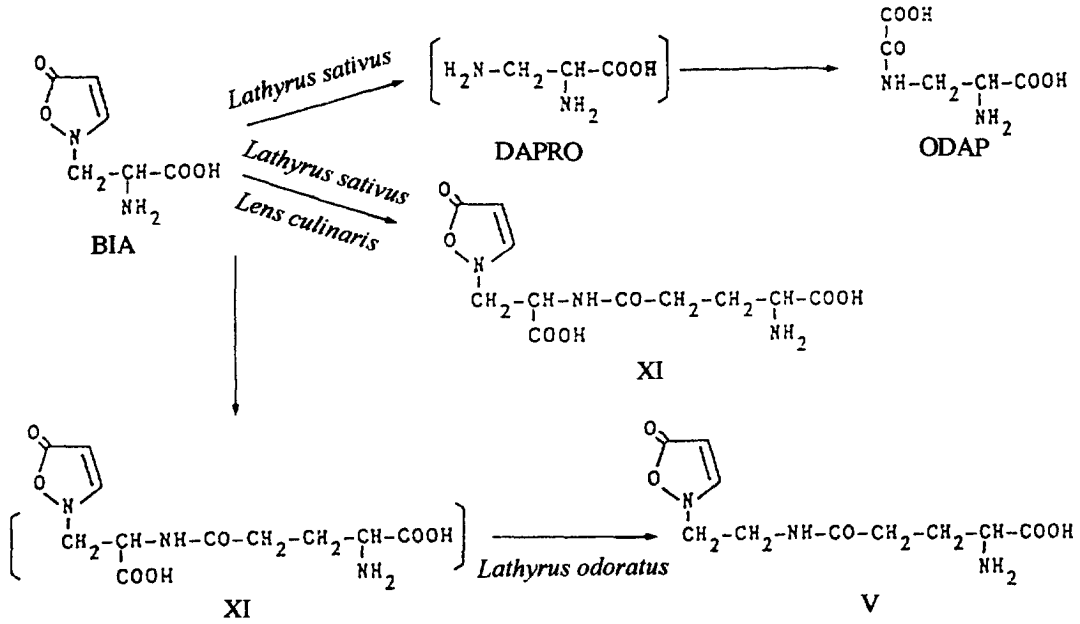
	Embryo	Cotyledon
Band XI	15.8%	16.1%
Band ACI (+V)	15.4%	16.1%

The above experiments suggest that in *L. odoratus* seedlings BIA is metabolised to compound V and it also suggests that compound XI is the short-lived intermediate between BIA and compound V.

We next initiated callus tissue of *L. odoratus* from leaf explants in B5 agar media and tried to confirm the biosynthesis of compound V by feeding the radioactive precursors [^{14}C]BIA or [^{14}C]XI to the calli. However, we could not identify ^{14}C-labelled compound XI nor compound V in the calli of *L. odoratus*. Previously, the feeding of [^{14}C]BIA of the calli of *L. sativus* showed only the formation of ^{14}C-labelled ODAP but not the formation of ^{14}C-labelled XI in the calli (before the feeding experiments, the secondary metabolites BIA, ODAP and XI were not detected in the habituated *L. sativus* calli used) [8]. These results indicate that enzyme(s) responsible for the biosynthesis of isoxazolinone compounds XI (γ-glutamyl transpeptidase) and V (decarboxylase) from BIA were not activated in habituated calli or that the relevant genes are not expressed in the calli under the condition used. The absence of secondary metabolites in habituated callus tissue is a common phenomenon [19].

The incorporation of [^{14}C]BIA in garden pea (*Pisum sativum*) seedlings and in lentil (*Lens culinaris*) seedlings were also studied by the same method. In 2-day-old *Pisum sativum* seedlings, about 50% of the total radioactivities in the 70% EtOH extracts was still in the band of BIA, in the embryo part, and about 30% in the band of BIA, in the cotyledon part. The other radioactive products seem to be the breakdown product of BIA. In 2-day-old *Lens culinaris* seedlings, [^{14}C]BIA was found to be metabolised to compound XI in the embryo part while in the cotyledons the concentration of XI was too low to show any radioactivity on autoradiography. Actually, the major radioactive compounds in both embryo and cotyledon of lentil seedlings were found to be four unknown compounds which are ninhydrin positive without UV-absorption (together about 30% of the total radioactivities in 70% EtOH extracts). These compounds were not identified.

The above investigations show that the metabolism of BIA in different legume seedlings is quite different, as summarised in Scheme 2. As toxin-free *L. sativus* might become an attractive protein-rich crop for drought prone and marginal lands, attempts to eliminate the toxin by genetic manipulation are being considered by several institutes [20]. The insertion of an alternative metabolic pathway for BIA into *L. sativus*, such as the biosynthesis of compound V from BIA (and XI) in *L. odoratus* can be considered. This way fewer molecules of BIA would be available to lead to



Scheme 2. Metabolism of BIA, β-(isoxazolin-5-one-2-yl)-alanine, in some legume seedlings. Brackets indicate that compound DAPRO (α,β-L-diaminopropionic acid) and compound XI (γ-glutamyl derivative of BIA) were not detected in *L. sativus* and in *L. odoratus* seedlings respectively, but are considered to be short-lived intermediates.

the biosynthesis of ODAP. However, it needs to be shown that compound V is not neurotoxic.

EXPERIMENTAL

Plant material

Seeds of *Lathyrus sativus* L. cv 8546 and cv 87041 were received from Dr C. G. Campbell (Agriculture Canada, Morden, Manitoba). Seeds of *Lathyrus odoratus* L. cv Spencer, *Lens culinaris* and *Pisum sativum* L cv Finale were from commercial sources.

Radioactive compounds

^{14}C-labelled L-BIA was prepared by *in vivo* incorporation of L-[U-^{14}C]serine (sp. act. 141 mCi mmol^{-1}, Amersham) in *Pisum sativum* seeds and extracted from 2-day-old seedling axes as before [6]. The sp. act. of [^{14}C]BIA was 10 μCi mmol^{-1}, and the label was exclusively present in the side-chain [8]. ^{14}C-labelled γ-glutamyl-L-BIA (compound XI) was prepared by *in vivo* incorporation of L-[U-^{14}C]serine (sp. act. 141 mCi mmol^{-1}, Amersham) in *L. sativus* 87041 seeds and extracted from 2-day-old seedling axes [2]. The sp. act. of [^{14}C]XI was 23 μCi mmol^{-1}. As in BIA, the label was in the alanyl-moiety of the side-chain. The purity of [^{14}C]BIA and [^{14}C]XI was checked with 2D TLC followed by autoradiography as before [6].

Feeding experiments

Surfaced-sterilised seeds of *L. sativus* L. cv 8546 were incubated with [^{14}C]BIA soln overnight. The imbibed seeds were rinsed several times with sterile dist. H_2O and grown in sterile vermiculite for 2 days at 25° in the dark. The embryo axes and cotyledon parts were harvested separately and 70% EtOH extracts were prepared and analysed by paper electrophoresis (105 min at 25 V cm^{-1}) as before. Under this condition the band of β-ODAP was well separated from the UV band containing BIA and compound XI. The elute of this UV band was subjected to 2D TLC (silica gel, Merck) to separate BIA and XI (Solvent 1: PhOH–H_2O, 3:1, solvent 2: *n*BuOH–HOAc–H_2O, 12:3:5).

Surface sterilised seeds of *L. odoratus* were incubated with [^{14}C]BIA soln or [^{14}C]XI soln overnight and grown for 3 days. Three-day-old embryo axes and cotyledons were extracted with 70% EtOH and analysed as above by paper electrophoresis. The band containing BIA was well separated from the UV band containing compound V and ACI. The elute of this UV band was subjected to 2D TLC to separate V and ACI.

Determination of radioactivity

The radioactivity of the purified compounds or the electrophoresis bands were determined in a liquid scintillation counter Wallac 1409 (LKB). The purity and the radioactivity of isolated ODAP, BIA, compound XI and V were confirmed in 2D TLC followed by autoradiography using authentic compounds as standards. All experiments were repeated 3–4 times. Typical examples are given in the tables.

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