Phytochemistry, 1978, Vol 17, pp. 1571–1576 © Pergamon Press Ltd. Printed in England 0031-9422/78/0901-1571 $02 00/0

BIOSYNTHESES OF THE URACILYLALANINES WILLARDIINE AND ISOWILLARDIINE IN HIGHER PLANTS*

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(*Received* 16 *December* 1977)

Key Word Index—*Pisum sativum*; *Lathyrus odoratus*; *Albizia julibrissin*; Leguminosae; *Fagus crenata*; Fagaceae; amino acids; pyridoxal phosphate catalysis; synthesis of willardiine; substrate inhibition.

Abstract—The syntheses of willardiine and isowillardiine were studied *in vivo* in pea seedlings by feeding uracil-[2-^{14}C] and *in vitro* with enzyme extracts from *Pisum*, *Lathyrus*, *Albizia*, *Leucaena* and *Fagus* seedlings by using uracil and *O*-acetyl-L-serine as substrates. The fate of isowillardiine in the intact seedlings has also been studied by feeding isowillardiine-[^{14}C] through the roots and determining its distribution. Some properties of willardiine and isowillardiine synthase(s) are described. Willardiine was also obtained by a B_6-catalysed chemical reaction under mild conditions. The question whether two enzymes are involved in the formation of the two isomeric uracilylalanines is discussed.

INTRODUCTION

Willardiine (**3**) has been isolated by Gmelin from seeds of *Acacia willardiana* [1]. It was subsequently identified in some 15 species of *Acacia* [2] and in some Mimosoideae [3, 4]. Its presence as a free amino acid, a γ-glutamylpeptide and a tripeptide in the seeds of some *Fagus* species has also been established recently [5]. The structure of willardiine has been confirmed by chemical synthesis in several laboratories [6–8].

The occurrence of isowillardiine (**4**) in pea seedlings was recognized concurrently but independently by Brown *et al.* [9] and by Lambein *et al.* [10]. Smaller amounts of willardiine were found in the same species [10]. The presence of isowillardiine in detectable amounts seems to be limited to the genus *Pisum*. While the chemical syntheses of β-uracil-4-ylalanine and β-uracil-5-ylalanine have been published some years ago [11], the synthesis of β-uracil-1-ylalanine (isowillardiine) was reported recently by Tsuchida *et al.* [12].

In *Pisum sativum* seedlings both willardiine [13] and isowillardiine [13, 14] have been shown to derive from uracil (**2**) and serine, probably via *O*-acetyl-L-serine (OAS) (**1**) (Scheme 1).

In the course of our ongoing work on the enzyme systems from higher plants capable of forming β-substituted alanines, we have demonstrated enzymatic syntheses of β-pyrazol-1-ylalanine [15], quisqualic acid [16], β-isoxazolinon-alanine derivatives [17, 18] and lupinic acid [19]. The *in vitro* biosynthesis of isowillardiine from uracil and OAS has been mentioned briefly by Ashworth *et al.* [13]. The present work sets out further evidence for the participation of OAS

* Part of this work was presented at the 97th Annual Meeting of the Pharmaceutical Society at Tokyo, 7 April 1977 (Abstracts II, p. 274).

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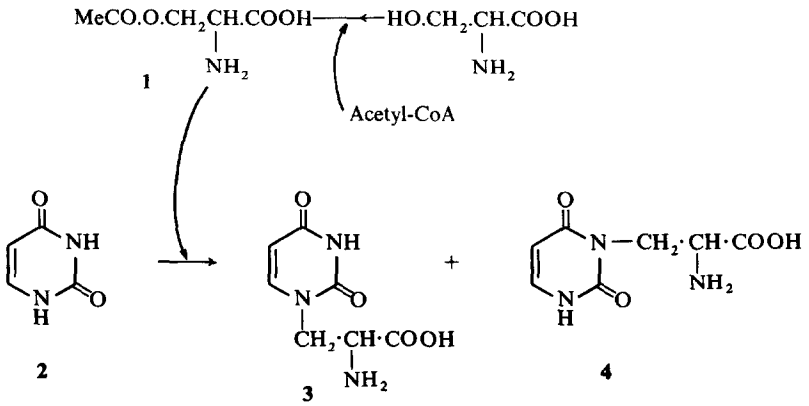

Scheme 1. Biosyntheses of willardiine (**3**) and isowillardiine (**4**) by enzyme(s) from seedlings of *Pisum sativum*, *Lathyrus odoratus*, *Albizia julibrissin*, *Leucaena leucocephala* and *Fagus crenata*.

in the formation of willardiine and isowillardiine and distribution of the enzyme(s) involved.

RESULTS

The occurrence of willardiine and isowillardiine synthase(s) and distribution within the plants

Cell-free systems were prepared from the seedlings of a number of *Pisum*, *Albizia*, *Lathyrus*, *Leucaena*, *Fagus*, *Citrullus* and *Lupinus* species. In general, willardiine and isowillardiine synthase(s) could be detected only in legume and *Fagus* seedlings where willardiine and isowillardiine occur naturally. The most active willardiine synthase preparations were obtained from *Lathyrus odoratus* seedlings (cotyledons removed): 51 nmol/mg protein/2 hr, vs 8 nmol/mg protein/2 hr for the isowillardiine synthase activity. A higher activity for isowillardiine synthase (21 nmol/mg protein/2 hr) than for willardiine synthase (18 nmol/mg protein/2 hr) was found only in preparations from *Pisum sativum* seedlings (cotyledons removed). Both activities were also found in *Albizia julibrissin*: 8 nmol **3** and 1 nmol **4**/mg protein/2 hr. Willardiine synthase alone was found in *Leucaena leucocephala* (4 nmol/mg protein/2 hr) and in *Fagus crenata* (0.3 nmol/mg protein/2 hr). Neither enzyme activity could be detected in *Fagus japonica*, *Lupinus luteus* or *Citrullus vulgaris* although analogous reactions for the syntheses of β-pyrazol-l-ylalanine [15] and lupinic acid [19] have been reported in *Citrullus* and *Lupinus* seedlings.

The willardiine and isowillardiine synthase activities increased during 3–8 days growth of *Pisum* seedlings, with increased contents of willardiine and isowillardiine, as shown in Figure. 1. Virtually no activity was found in extracts of 1-day-old seedlings. The specific activities of the willardiine and isowillardiine synthases prepared from 8-day-old seedlings (cotyledons removed) of *Pisum sativum* were approximately 12 and 100 fold greater, respectively, than that from the cotyledons as shown in Fig. 1. A relatively higher activity of willardiine synthase is present in the cotyledons.

The identity of the reaction products as willardiine and isowillardiine was confirmed by their chromatographic behaviour on PC and their positions of elution from an automatic amino acid analyser and by comparison with natural and synthetic materials. Further confirmation of the enzyme catalysed formations of willardiine and isowillardiine was also determined by feeding OAS-[3-^{14}C] or uracil-[2-^{14}C] as substrates.

For routine assay of the enzyme activities, an automatic amino acid analyser was employed which allowed quantitative determinations: under standard operating condition, willardiine was eluted from the column at a position close to an aspartic acid reference peak, whilst isowillardiine appeared at a position of *S*-methylcysteine close behind proline.

Using an amino acid analyser coupled to a [^{14}C]-monitoring flow system [15], we found that when a complete incubation mixture with added OAS-[3-^{14}C] or uracil-[2-^{14}C] was examined, radioactive peaks clearly corresponded with the ninhydrin peaks of both willardiine and isowillardiine. In the enzyme blanks no radioactivity was observed at the positions corresponding with willardiine or isowillardiine. The reaction mixtures were also applied on PC where willardiine and isowillardiine could be located in UV light and where the radioactivity was located with a chromatoscanner.

Properties of willardiine and isowillardiine synthase(s)

In all cases enzyme preparations were capable of alanylating uracil (**2**) in the presence of OAS (**1**) as a donor of the alanyl moiety. The willardiine and isowillardiine synthase(s) clearly appear to be specific for OAS as a substrate: *O*-acetyl-D-serine or other esters of L-serine, such as the phosphate and sulfate, or L-serine itself could not serve as donor molecules. The enzyme(s) was reasonably stable: when stored at 0° for 24 hr, the residual enzyme activities for willardiine

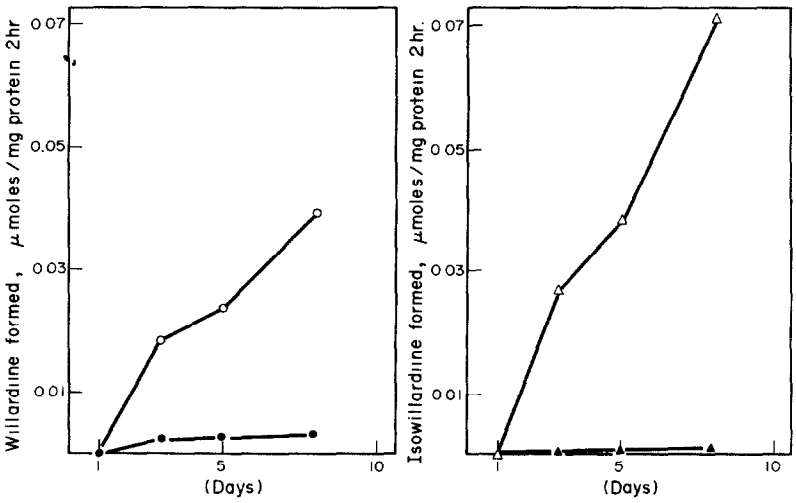

Fig. 1. Specific activities for the willardiine (○) and isowillardiine (△) synthase(s) in extracts from the seedlings (cotyledons removed) (○, △) and the cotyledons (●, ▲) of etiolated *Pisum* seedlings during germination.

and isowillardiine formation were about 89 and 63% respectively of initial activities.

The effect of pH on the rates of *N*-1 and *N*-3-alanylation of uracil by the enzyme(s) was investigated by using 0.1 M K-Pi buffers. Synthesis of both products was maximal at pH 7.8–7.9 although there is a rapid *O*-acetyl to *N*-acetyl shift in the substrate above about pH 8.0. Both activities were half maximal at pH 7.5 and 8.2.

When the reaction mixtures were incubated at 30° for varying periods up to 3 hr, the rates of willardiine and isowillardiine formation were related linearly with time for at least 60 min and then the rates gradually decreased.

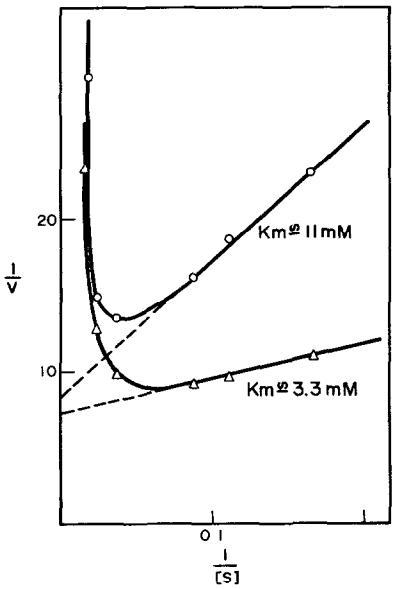

Fig. 2. Influence of increasing concentrations of uracil on the enzymic synthesis of willardiine (○) and isowillardiine (△) plotted according to Lineweaver–Burk.

The synthase activities for willardiine and isowillardiine were dependent upon the concentration of uracil used. A relatively low final concentration of uracil at around 13 mM and 35 mM was sufficient to give maximum rates of willardiine and isowillardiine formation respectively. In both cases higher concentrations of uracil lead to progressively more marked substrate inhibition. The Lineweaver–Burk plot illustrates this clearly (Fig. 2). The apparent K_m values are 11 mM for willardiine and 3.3 mM for isowillardiine.

Willardiine and isowillardiine synthase(s) show increased activity when pyridoxal phosphate is added as a coenzyme. Maximum reaction rates were observed in mixtures containing about 60 μg/ml of exogenous pyridoxal phosphate but a higher concentration (250 μg/ml) caused slight inhibition of the synthase activities: 12% for the willardiine synthase and 4% for the isowillardiine synthase. The rates of willardiine and isowillardiine formation were stimulated by additions of pyridoxal phosphate at 60–63 μg/ml about 2.8 fold and 3.2 fold, respectively, over mixtures containing no added pyridoxal phosphate. Complete inactivation of the enzyme(s) was achieved with hydroxylamine and KCN at concentrations of 100 mM in each case.

Chemical synthesis of willardiine

Willardiine has also been synthesized chemically by incubating a 0.1 M acetate buffer solution containing uracil and OAS or serine at pH 5.5 in the presence of pyridoxal phosphate and gallium ions at 35° for 2–3 hr but the formation of isowillardiine was negligible under the same conditions. Detailed results on the chemical syntheses of heterocyclic β-substituted alanines by B_6-catalyzed chemical reactions will be reported later.

Biosyntheses of willardiine and isowillardiine in vivo

The incorporation of uracil-[2-^{14}C] into willardiine and isowillardiine *in vivo* was studied by two methods. In the first method the dry seeds of *Pisum sativum* were surface sterilized and allowed to imbibe a solution of uracil-[2-^{14}C] for 17 hr and the seeds were then germinated in sterilized vermiculite for up to 14 days. In the second method the seeds were germinated in sterilized vermiculite. After a certain germination period the whole seedlings were immersed in a solution of uracil-[2-^{14}C] under vacuum, after 30 min the vacuum was gradually released and after another 1 hr of immersion time the seedlings were germinated for a further 24 hr in sterile vermiculite.

At regular intervals a number of seedlings were harvested, and the total and specific radioactivities of

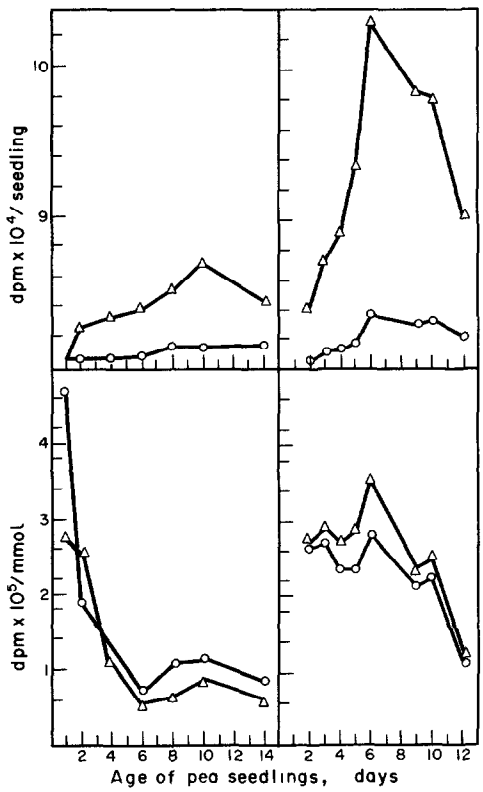

Fig. 3. Incorporation of uracil-[2-^{14}C] into willardiine (○) and isowillardiine (△) in *Pisum* seedlings, by two methods. Left: uracil uptake during imbibition; right: uptake by soaking under vacuum 24 hr before extraction (see Experimental for details). The upper graphs show the radioactivity in the two isomers per seedling. The lower graphs show the specific radioactivity of purified compounds.

Table 1. Distribution of isowillardiine-[^{14}C] in *Pisum* seedlings grown in the dark. The roots of 6-day-old seedlings were immersed in 5 ml isowillardiine-[^{14}C] solution (273 × 10^9 dpm/mol) during 24 hr and then grown further for the indicated period

	1 day			4 days		
	%dpm	dpm/g fr. wt	dpm/μmol	% dpm	dpm/g fr. wt	dpm/μmol
Plumule	13.7	34140	17570	12.5	13970	18440
Epicotyl	3.4	9910	5325	5.4	20060	5940
Root	78.1	127350	46200	76.8	71580	40750
Cotyledons	4.8	2990	9960	5.3	3870	15880
Total	91900	33900		97000	27610	

willardiine and isowillardiine were determined by sequential PC of the 70% ethanol extracts of the seedlings (cotyledons removed). The results are presented in Fig. 3.

When the incorporation of uracil-[2-^{14}C] was studied with the seedlings of *Lathyrus odoratus* (sweet pea) by the imbibition method, extremely small amounts of labelled isowillardiine were isolated (unlabelled isowillardiine was added to the crude extract). As compared to the concentration of isowillardiine in pea seedlings, the concentration in sweet pea seedlings was 5 × 10^5 fold less.

Uptake of isowillardiine-[^{14}C] by seedlings

Labelled isowillardiine was prepared from the samples of the *in vivo* experiments. Aseptically grown 6-day-old *Pisum* seedlings were allowed to take up the labelled isowillardiine by immersing the roots in a solution during 24 hr at 25° in the dark and were then transferred to vermiculite and further grown in the dark. After 1 day or 4 days (8- or 11-day-old seedlings), the seedlings were cut into 4 parts and extracted separately with 70% EtOH. Willardiine and isowillardiine were purified by sequential PC, and the total and specific activity of isowillardiine was determined (Table 1). Willardiine was found to be unlabelled in each case, in agreement with Ashworth *et al.* [13].

In another experiment 4-day-old seedlings were used: after uptake of labelled isowillardiine they were grown in normal daylight for 7 or 14 days (12- and 19-day-old seedlings), and were analysed as before (Table 2). After an initial period of uptake and translocation, no further net movement of label occurs. The major part of the isowillardiine-[^{14}C] taken up by the seedlings could be recovered unchanged. *Pisum* seedlings seem to readily take up isowillardiine through the roots, while it is also exuded from the roots [22]. Inside the seedlings the major direction of translocation of isowillardiine is from the roots to the plumule. The role of the cotyledons as a sink for this metabolically stable product is very limited.

DISCUSSION

From the above results it can be concluded that some seedlings of higher plants contain enzymes capable of substituting the free uracil ring at nitrogen with an alanyl side chain and that this side chain is derived from *O*-acetyl-L-serine. Free uracil is present in pea seedlings [23] and in other higher plants [24] but not in the dry peas [25]. In the case of the *Pisum* enzyme, the activities of willardiine and isowillardiine synthase(s) are stimulated by addition of pyridoxal phosphate.

In the case of intact seedlings of *Lathyrus odoratus*, this enzyme(s) seems to be inactive towards uracil *in vivo* but becomes active after purification from low molecular weight material. However the isoxazolin-5-one ring is substituted into β-isoxazolin-5-on-2-yl-alanine in the *Lathyrus odoratus* seedlings as well as in its extracts. The apparent specificity of this enzyme might be due to the presence of a specific inhibitor.

Our results suggest the presence of two enzyme systems, each catalysing the formation of only one of the isomeric β-uracilyl-alanines. Thus the two enzyme activities show differences in distribution, in stability, in optimal substrate concentration and in K_m. Nevertheless, they are similar in substrate requirements, and show a dependence upon pyridoxal phosphate to an almost identical degree; furthermore, both enzyme activities have identical pH optima and are inactivated by the same inhibitors.

Table 2. Distribution of isowillardiine-[^{14}C] in *Pisum* seedlings grown under normal day light. The roots of 4-day-old seedlings were immersed in 5 ml isowillardiine-[^{14}C] solution (235 × 10^9 dpm/mol) during 24 hr in the dark and then grown further for the indicated period under daylight (2 × 5 seedlings/expt)

	7 days			14 days		
	% dpm	dpm/g fr. wt	dpm/μmol	% dpm	dpm/g fr. wt	dpm/μmol
Plumule	10.5	9208	14900	11.0	7330	16700
Epicotyl	5.3	11990	11700	4.3	12020	7500
Root	77.7	40220	58400	79.6	36070	45300
Cotyledons	6.5	4200	20900	5.1	4340	15600
Total	312400	18820		352900	5460	

EXPERIMENTAL

Plant materials. The seedlings of pea (*Pisum sativum*), watermelon (*Citrullus vulgaris*), *Albizia julibrissin*, *Leucaena leucocephala*, and *Lupinus luteus* were grown in moistened vermiculite in the dark for 5–6 days at 30°, sweet pea (*Lathyrus odoratus*) seedlings for 6–8 days at 30°, and seedlings of *Fagus crenata* and *F. japonica* for 10–14 days at 30°, After harvest, the cotyledons were removed and the seedlings were cooled at 0° for 30 min before enzyme extraction.

Chemicals. OAS-[3-^{14}C] was synthesized in our laboratory from L-serine-[3-^{14}C] by a modification of the method of Sheehan *et al.* [20] and diluted with unlabelled OAS to obtain material of sp. act. used in the different incubation mixtures. Willardiine (**3**) and isowillardiine (**4**) were obtained from *Pisum* seedlings, and a synthetic sample of **4** was a gift from Prof. Y. Mizuno, Drs. K. Ikeda and K. Tsuchida. **4**-[^{14}C] was isolated from pea seedings after uptake of uracil-[2-^{14}C] and purified as described [10].

Enzyme preparations. All operations were carried out at about 4°. The enzyme prep. were obtained from the whole seedlings, the embryo or the cotyledons as described [15–19]: unless otherwise stated, the seedlings (cotyledons removed) (50 g) were homogenized in a mortar with a little Si sand and 0.2 M KPi buffer, pH 7.5, containing 0.5% 2-SH-EtOH (2 ml/g tissue).

The clear supernatant, recovered by centrifugation at 25000 *g* for 30 min, was immersed in a H_2O bath at 80° and stirred vigorously until the temp. of the solution reached 55°. It was then immediately chilled to 4° with ice water, and centrifuged at 25000 *g* for 15 min. The resulting solutions were subjected to $(NH_4)_2SO_4$ fractionation, and the ppt obtained between 30 and 70% satn were dissolved in 5.0 ml 0.1 M KPi buffer, pH 7.5. These clear protein solutions, after centrifugation at 25000 *g* for 15 min, were applied to a column of Sephadex G-25 (fine) equilibrated with 0.1 M KPi buffer, pH 7.5, and the same buffer was used to elute the protein fraction and to remove the low MW substances. The eluted protein fractions were used immediately in the following experiments as the source of enzyme. Protein was determined by the method of Lowry *et al.* [21].

Assay for willardiine and isowillardiine synthase activities. Reaction mixtures used to demonstrate the formation of **3** and **4** were conducted at 30° in a final vol. of 1.1 ml and contained OAS or OAS-[3-^{14}C] (10 μmol, 1.0 μCi) uracil or uracil-[2-^{14}C] (20 μmol, 1.42 μCi) and 0.5 ml enzyme prep. containing 2–5 mg of the protein corresponding to *ca* 5 g fr. wt of plant tissue. The pH of the incubation mixtures was normally adjusted to pH 7.8 by 0.1 M KPi buffer. The reaction was terminated by addition of 3 vol. EtOH. The protein ppt was removed by centrifugation and the clarified supernatant was examined chromatographically for the presence of **3** and **4**.

Occasionally, OAS was replaced by *O*-acetyl-D-serine, L-serine-*O*-sulfate, *O*-phospho-L-serine and L- or D-serine as a donor of the alanyl moiety. NH_2OH and KCN as inhibitors, and pyridoxal–Pi as a coenzyme were added to certain reaction mixtures.

PC was performed in the following solvents: 1,*i*-PrOH–HCO_2H–H_2O (20:3:8); 2,*t*-BuOH–HCO_2H–H_2O (5:1:1); 3.*n*-PrOH–EtOH–H_2O (3:7:9). The R_fs for **3** obtained in these solvents were 0.29, 0.26 and 0.53 respectively; **4** had the following R_f: 0.36, 0.33 and 0.45 respectively. Under the same conditions, serine had R_fs 0.52, 0.44 and 0.61 and OAS R_fs: 0.66, 0.60 and 0.70 respectively.

Further confirmation of the identity of the reaction products as **3** and **4** was obtained by using an automatic amino acid analyser (Shibata Model AA-500, Tokyo or Jeol LJC-5AH). Under standard operating conditions (150 cm column, 50°, 0.2 N Na-Ci buffer, pH 3.25, flow rate 0.5 ml/min), **3** eluted at about 112 ml, i.e. at a position close to asp. ref. peak, whilst **4** appeared at a position (200 ml) of *S*-Me-cysteine ref. peak. However, since **3** slightly overlapped with the OAS peak, the reaction mixtures were allowed to stand at pH 9.5 (dil. NH_4OH) for 60 min at room temp. before applying the samples to an amino acid analyser: by this procedure OAS was almost quantitatively rearranged into *N*-acetylserine.

When unlabelled OAS or uracil was replaced in the reaction mixture with OAS-[3-^{14}C] or uracil-[2-^{14}C], radioactivity associated with each amino acid peak was recorded with a Packard monitoring flow system, model 3002, coupled to a ratemeter, model 282A. Radioactivity on the PC was monitored with a gas-flow 4 π-radiochromatogram scanner (Aloka model PCB-2B, Tokyo). **3** and **4** obtained from *in vivo* expts were identified with PC in 4 solvents, automatic amino acid analyser and 2-dim. TLC coupled with autoradiography.

For routine quantitative det. of **3** and **4** formation, the automatic amino acid analyser was employed, allowing accurate determinations.

Media for the incorporation expts. For the imbibition of surface sterilized seeds, the dry *Pisum* seeds were soaked in uracil-[2-^{14}C] soln (100 μCi/150 ml) for 17 hr at room temp., *ca* 73% of radioactivity was taken up. For the soaking of seedlings 50 μCi uracil-[2-^{14}C] was dissolved in 100 ml Key medium. Seedlings of 2, 3, 4, 5, 6, 9, 10 and 12 days old were used. After removing the testa the seedlings were completely immersed in the soln and placed *in vacuo* for 30 min, then air was allowed to enter slowly (*ca* 10% of the act. was taken up). The seedlings were removed from the soln after another 60 min and transferred to vermiculite for 24 hr. The cotyledons were removed before extraction.

For the incorporation of **4**-[^{14}C] in the dark (Table 1) 6 seedlings (6 days old) were rinsed with H_2O and the roots (not the cotyledons) were immersed in 5 ml soln containing 1.5×10^6 dpm (sp. act. 273×10^9 dpm/mole **4**). After 24 hr at 25° 14.2% of the activity was taken up. The seedlings were transferred to vermiculite in the dark for further 1 or 4 days growth. The plantlets were then cut into 4 parts (roots, cotyledons, epicotyl and plumule) and extracted with 70% EtOH. **3** and **4** were purified by sequential PC in EtOH–H_2O (4:1), *i*-PrOH–H_2O (7:3) and *n*-BuOH–HOAc–H_2O (12:3:5). Radioactivity was measured with a Packard Tri Carb liq. scint. spectrometer (mod. 544). Normally 50 μl aq. sample in 5 ml dioxane based scintillator.

For the incorporation of **4**-[^{14}C] under day light 2×10 seedlings (4 days old) were immersed with the roots in 5 ml H_2O containing 1.7×10^6 dpm (235×10^9 dpm/mol **4**). After 24 hr at 25°, 29.2 and 29.6% respectively of the activity was taken up. After germinating in vermiculite under day light (June) for 7 or 14 days, half of each batch was harvested and analysed as above (Table 2).

Acknowledgements—We are grateful to Prof. L. Fowden, Harpenden, for his encouragement and to Prof. Y. Mizuno, Drs K. Ikeda and K. Tsuchida, Faculty of Pharmaceutical Sciences, Hokkaido University, Japan, for a gift of synthetic (±)-isowillardiine. We also are indebted to Mr Y. Yamaga, National Forest Office at Inawashiro, Japan, for cordially supplying *Fagus* seeds and to Mr T. Ikada and his students in the Scientific Group of Okinoerabu High School, Kagoshima, Japan, for kindly providing *Leucaena* seeds. Y.-H. K. received a fellowship from the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek. F.L. acknowledges a personal grant from the same institution.

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