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INTERMEDIATES IN THE BIOSYNTHESIS OF LINAMARIN

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Abstract—Linen flax shoots were examined under conditions designed to promote the accumulation of intermediates of linamarin biosynthesis. Experiments with unlabelled isobutyraldoxime and isobutyronitrile administered together with L-valine-U-^{14}C showed that both these compounds could be intermediates. DL-*o*-Methylthreonine, DL-*allo*-*o*-methylthreonine and DL-2-methoxypropionaldoxime were found to be inhibitors of linamarin biosynthesis and to cause accumulation of a hitherto undetected compound, which may be isobutyraldoxime-*o*-β-glucoside. Accumulation of isobutyraldoxime was also demonstrated in some inhibitor treatments.

INTRODUCTION

EXPERIMENTS with labelled precursors have indicated that isobutyraldoxime, isobutyronitrile and 2-hydroxyisobutyronitrile may be intermediates in the biosynthesis of linamarin (2-hydroxyisobutyronitrile β-D-glucoside) from L-valine in flax (*Linum usitatissimum* L.).[1] Before a compound may be established as an intermediate it is necessary to demonstrate its formation *in vivo*, its utilization, and to isolate the enzymes involved in its metabolism.

Attempts to demonstrate the presence of the proposed intermediates in extracts of the untreated shoots of flax were not successful, possibly because of insufficient sensitivity for the detection of very small amounts of the intermediates. Alternative methods were used in the experiments described in this paper.

If a sample of the compound proposed as an intermediate is administered along with a labelled precursor then the labelled intermediate may be diluted and accumulate in the tissue. The use of this type of procedure has been described as 'trapping'. Alternatively, accumulation of an intermediate may be induced by administering a specific inhibitor of the biosynthesis. In both cases a reduction of incorporation of label into the final product is expected and further, an intermediate compound may act as an inhibitor under unphysiological administration conditions. This paper describes experiments using both techniques to demonstrate the presence of possible intermediates in the biosynthesis of linamarin.

RESULTS

Survey of Inhibitors of Linamarin Biosynthesis

In an attempt to inhibit linamarin biosynthesis and induce the accumulation of possible intermediates a range of analogues of valine was administered along with L-valine-U-^{14}C to linen flax shoots (Table 1). Extracts of the seedlings were qualitatively examined by two-dimensional paper chromatography. The strong inhibition of linamarin biosynthesis by the DL-*o*-methylthreonine was selected for further study as this treatment, like the *allo* isomer treatment, also induced the considerable accumulation of a hitherto unobserved radioactive

[1] B. A. TAPPER and G. W. BUTLER, *Biochem. J.* (in press).

TABLE 1. SURVEY OF INHIBITORS OF LINAMARIN BIOSYNTHESIS

Compound*	μmoles	Inhibition	Compound I
L-*allo*-Isoleucine	5	Nil	Nil
2-Hydrazinoisovaleric acid	5	Nil	Nil
DL-*erythro*-2-Amino-3-chlorobutyric acid	5	Nil	Nil
DL-*threo*-2-Amino-3-chlorobutyric acid	5	Nil	Nil
2-Amino-3-ethylvaleric acid	5	Strong	Nil
DL-2-Amino-3-methiobutyric acid	5	Strong	Nil
DL-*o*-Methylthreonine	5	Strong	Considerable
DL-*allo*-*o*-Methylthreonine	5	Strong	Considerable
Isobutylamine hydrochloride	10	Weak	Trace
2-Methoxypropionamine hydrochloride	10	Weak	Moderate

* Compounds were administered together with L-valine-U-^{14}C (1 μmole) to 20 flax shoots for 7 hr under artificial light.

compound, I. This compound had R_fs a little greater than linamarin in all paper and thin layer chromatography systems used.

Table 2 contains results of experiments where the inhibitory action of DL-*o*-methylthreonine is compared with some other compounds of related structure. DL-2-Methoxypropionaldoxime was inhibitory and also induced the accumulation of compound I in a similar way to DL-*o*-methylthreonine. DL-3-Methoxyvaline and DL-2-methoxypropionitrile may have inhibitory action at other sites in the linamarin biosynthesis pathway as compound I did not accumulate, or may be more generally toxic to the flax seedling shoots.

TABLE 2. INHIBITION OF LINAMARIN BIOSYNTHESIS AND FORMATION OF COMPOUND I FOLLOWING ADMINISTRATION OF L-VALINE-U-^{14}C AND OTHER COMPOUNDS

Expt.	L-Valine-U-^{14}C (μmoles)	Inhibitor	Amount (μmoles)	Incorporation* from L-Valine-U-^{14}C: % Converted to linamarin	% Converted to I
1	1·2		—	23	Nil†
	1·2	DL-*o*-Methylthreonine	10	9	3
	1·2	DL-3-Methoxyvaline	10	10	Nil†
2	1·0	DL-*o*-Methylthreonine	10	7	3
	1·0	DL-2-Methoxypropionaldoxime	9	5	4
	1·0	DL-2-Methoxypropionitrile	10	2·5	Nil†

* Assuming a loss of carboxy-^{14}C from L-valine-U-^{14}C.
† Estimated as less than 0·5% which was the minimum detectable level on radioautographs.

Volatile Intermediates

Although the use of GLC failed to produce evidence for isobutyraldoxime or isobutyronitrile in untreated flax seedlings, the biosynthesis of both compounds could be demonstrated by trapping experiments where L-valine-U-^{14}C was administered along with the unlabelled aldoxime or nitrile. Isobutyraldoxime could also be detected in seedling shoots following treatment with DL-*o*-methylthreonine and DL-2-methoxypropionaldoxime.

TABLE 3. RADIOACTIVITY IN THE ISOBUTYRALDOXIME FRACTION FOLLOWING ADMINISTRATION OF L-VALINE-U-^{14}C AND OTHER COMPOUNDS

Treatment	Amount (μmoles)	Dis/min in isobutyraldoxime fraction
Control	—	65
Isobutyraldoxime	60	1640
DL-*o*-Methylthreonine	10	5690

Table 3 records the radioactivity recovered in the GLC fractions containing carrier isobutyraldoxime following the treatment of batches of 20 flax seedling shoots with L-valine-U-^{14}C (2 μmole; 1 μc) in the presence of unlabelled isobutyraldoxime or DL-*o*-methylthreonine. The shoots were exposed for 8 hr. The results show an appreciable accumulation of radioactivity in two of the treatments compared with the control where L-valine-U-^{14}C alone was administered. The presence of isobutyraldoxime-^{14}C was confirmed by treating portions of the extract with 2,4-dinitrophenylhydrazine in dilute acid to give isobutyraldehyde 2,4-dinitrophenylhydrazone which was identified by TLC.

In a subsequent experiment L-valine-U-^{14}C (1 μmole; 2 μc) was administered with treatments of unlabelled isobutyraldoxime, isobutyronitrile, methacrolein oxime, methacrylonitrile and DL-2-methoxypropionaldoxime at concentrations indicated in Table 4.

TABLE 4. RADIOACTIVITY WITH A RETENTION TIME THE SAME AS ISOBUTYRALDOXIME FOLLOWING ADMINISTRATION OF L-VALINE-U-^{14}C AND OTHER COMPOUNDS

Treatment	Amount (μmoles)	Net counts
Control	—	80
Isobutyraldoxime	20	3150
DL-2-Methoxypropionaldoxime	10	1510
Isobutyronitrile	60	120
Methacrylonitrile	24	150
Methacrolein oxime	20	700

Exposure to the L-valine-U-^{14}C was for 10 hr. Results were compared with a control where only L-valine-U-^{14}C was administered. In this experiment a major portion of the effluent of the diethylene glycol succinate column was passed through a gas flow proportional counter.

Upon GLC, the treatments with isobutyraldoxime, DL-2-methoxypropionaldoxime and methacrolein oxime all gave significant peaks of radioactivity with a retention time the same as isobutyraldoxime of 20 min and with the amounts of radioactivity decreasing in that order. The relative amounts of radioactivity detected are given in Table 4 for all the treatments and the control. The figures are the net counts recorded for the 1·6 min interval during which isobutyraldoxime eluted from the column. The absolute efficiency of the method was not determined. Other peaks of radioactivity appeared in varying amounts but were also present in the control. The large peak of radioactivity with retention time of 20 min from the isobutyraldoxime treatment was collected and identified by the preparation

of isobutyraldehyde 2,4,-dinitrophenylhydrazone. The corresponding radioactive peaks for the other two treatments of DL-2-methoxypropionaldoxime and methacrolein oxime are presumed to be isobutyraldoxime. The methacrolein oxime had a retention time of 24 min and was unlabelled.

The treatment containing isobutyronitrile gave a chromatogram similar to the control with peaks of radioactivity with retention times of 5, 6 and 10·5 min. This treatment also contained a significant peak of radioactivity of 10 min not present in the control. This peak, although not completely resolved from a peak at 10·5 min, was collected, diluted with pure isobutyronitrile and *tert*-butylisobutyramide prepared from it. Recrystallization to constant specific activity indicated that approximately 70% of the radioactivity trapped in the peak was isobutyronitrile.

The effect of administration of methacrylonitrile and methacrolein oxime was examined as both of these compounds could, in theory, be intermediates in linamarin biosynthesis, in which case an accumulation of radioactivity in them would have been expected. The results of GLC did not, however, give any evidence for radioactivity in either of the methacryl compounds.

Properties and Structure of Compound I

The structure proposed for compound I is isobutyraldoxime-*o*-β-D-glucopyranoside. The presence of the glucose moiety was demonstrated by administering D-glucose-U-^{14}C together with L-valine and DL-*o*-methylthreonine to flax seedling shoots. Following treatment of the compound I from paper chromatograms with β-glucosidase, labelled glucose was re-isolated.

Radioactive compound I prepared from L-valine-U-^{14}C was used to investigate the nature of the aglycone moiety. Treatment of compound I with 0·5 N HCl for 20 hr at 40° yielded radioactive isobutyraldehyde while treatment with β-glucosidase gave radioactive isobutyraldoxime. Pyrolysis yielded radioactive isobutyronitrile which was identified by conversion to *tert*-butylisobutyramide and recrystallization to constant specific activity.

An attempt to demonstrate metabolism of compound I by administering the radiochemically pure substance back to flax shoots was not successful. The seedling shoots were held in the light for 7 hr. No other radioactive compounds were detected on paper chromatograms of the extracts.

DISCUSSION

The experiments described here show that isobutyraldoxime is synthesized in detectable amounts from L-valine by flax shoots in the presence of certain inhibitors and in trapping experiments, although it was not detected in untreated shoots. In view of the substantial incorporation of isobutyraldoxime,[1,2] it may be accepted as a natural intermediate in linamarin biosynthesis.

The accumulation of radioactive isobutyraldoxime and radioactive compound I, when either DL-*o*-methylthreonine or DL-2-methoxypropionaldoxime is administered with L-valine-U-^{14}C suggests that the specific site of inhibition of linamarin biosynthesis by these compounds is the conversion of isobutyraldoxime to isobutyonitrile or some other intermediate.

[2] B A. TAPPER, E. E. CONN and G. W. BUTLER, *Arch. Biochem. Biophys.* **119**, 593 (1967).

The simplest structure for compound I consistent with the observed properties is isobutyraldoxime-*o*-β-D-glucopyranoside. This structure would account for the hydrolysis to isobutyraldehyde in dilute acid and to isobutyraldoxime with β-glucosidase. The decomposition of aldoxime *o*-derivatives to nitriles is known and would account for the formation of isobutyronitrile upon pyrolysis. Compound I may be a by-product formed in detectable amounts only when the accumulation of isobutyraldoxime is induced; alternatively, it may be an intermediate.

The accumulation of radioactive isobutyronitrile when the unlabelled compound was administered in considerable quantities along with L-valine-U-^{14}C indicates this compound may well be a natural intermediate in linamarin biosynthesis, even although the effect was less than in the case of isobutyraldoxime.

EXPERIMENTAL

Materials. L-Valine-U-^{14}C and D-glucose-U-^{14}C were obtained from the Radiochemical Centre, Amersham. 2-Oximinoisovaleric-U-^{14}C acid was prepared as previously described.[2]

DL-*o*-Methylthreonine and DL-*allo*-*o*-methylthreonine were prepared by the method of West and Carter.[3] DL-2-Methoxypropionaldoxime was prepared by reacting DL-*o*-methylthreonine (0·5 g) and ninhydrin (2 g) in 20 ml of pH 2·5, 1 M citrate buffer at 100° for 10 min. The resulting DL-2-methoxypropionaldehyde was then steam distilled off and the distillate treated with hydroxylamine hydrochloride (1 g) and $NaHCO_3$ (1·5 g). The oxime, formed after 24 hr at room temp., was extracted into Et_2O and purified by GLC. DL-Methoxypropionitrile was prepared by the method of Adams *et al.*[4] and DL-3-methoxyvaline was made by the procedure of West *et al.*[5]

Methacrylonitrile was prepared by heating methacrylamide with excess P_2O_5. The crude nitrile was distilled off and redistilled to give a fraction with b.p. 88–89° (745 mm). Methacrolein oxime obtained by treatment of methacrolein with hydroxylamine hydrochloride in aqueous Na_2CO_3 was distilled under reduced pressure (76–78°/45 mm).

L-*allo*-Isoleucine, 2-hydrazinoisovaleric acid, DL-*erythro*-2-amino-3-chlorobutyric acid, DL-*threo*-2-amino-3-chlorobutyric acid, 2-amino-3-ethylvaleric acid and DL-2-amino-3-methiobutyric acid were gifts from Dr. M. Rabinowitz, National Cancer Institute, Md., U.S.A.

Administration of labelled compounds, extraction and analysis. Labelled compounds, in some cases accompanied by possible inhibitors or competitors, were administered to excised shoots of linen flax seedlings which had been germinated on moist cotton wool at 25° in darkness for four days followed by 18 hr exposure to artificial light.[1,6] In a typical experiment the compounds were dissolved in water (200 μl) for administration to 20 seedling shoots. Additional water was added as required. Where the shoots were examined for non-volatile compounds they were extracted and analysed by methods already described.[1]

Variations of procedure were used when the tissue was examined for volatile radioactive compounds at the end of the experiments. In the first experiment L-valine-U-^{14}C (2 μmole, 1 μc) together with unlabelled isobutyraldoxime (60 μmoles) or DL-*o*-methylthreonine (10 μmoles) or by itself was administered to batches of 20 seedling shoots. All treatments were enclosed in 200 ml transparent containers for 2 hr and exposed to light for 8 hr after which the shoots were blended with cold Et_2O (50 ml) for 1 min. 5 ml portions of the ether extracts were concentrated in the presence of added carrier isobutyraldoxime prior to separation on a Carbowax 400 column.

In the second experiment L-valine-U-^{14}C (1 μmole, 2 μc) was administered to batches of 20 flax seedling shoots with treatments of unlabelled isobutyraldoxime, isobutyronitrile, methacrolein oxime, methacrylonitrile, and DL-2-methoxypropionaldoxime at concentrations indicated in Table 4. The last compound, being considered non-volatile, was administered with the L-valine-U-^{14}C in 140 μl H_2O. Two portions of H_2O (50 μl) were added during the first 2·5 hr after which the shoots were enclosed in 12 ml glass vessels. The other treatments were similar except that the unlabelled compounds were administered after the first 2·5 hr which allowed prior uptake of virtually all of the L-valine-U-^{14}C solution. Volatile compounds were probably assimilated from the vapour phase. A further 7·5 hr was allowed for metabolism of the L-valine-U-^{14}C in the presence of the added unlabelled compounds. Results were compared with a control where only L-valine-U-^{14}C was used. The shoots were extracted by grinding together with 1·5 ml Et_2O, 1·5 g of 4 mm

[3] H. D. West and H. E. Carter, *J. Biol. Chem.* **119**, 109 (1937).
[4] R. Adams, C. C. J. Culvenor, C. N. Robinson and H. A. Stingl, *Austral. J. Chem.* **12**, 706 (1959).
[5] H. D. West, G. S. Krummel and H. E. Carter, *J. Biol. Chem.* **122**, 605 (1937).
[6] G. W. Butler and E. E. Conn, *J. Biol. Chem.* **239**, 1674 (1964).

glass beads and 0·5 g of anhydrous Na_2SO_4 in small vessels on a high speed shaker. The extracts were filtered by centrifugation into small vials and about 1 ml recovered. Samples (150 μl) of these extracts were taken for GLC on a diethylene glycol succinate column.

Gas chromatography. Volatile compounds were investigated by GLC using either 15% (w/w) Carbowax 400 on Chromosorb W (60–80 mesh, DMCS treated) packed in a 3 m × 7·6 mm i.d. aluminium column or 21% (w/w) diethylene glycol succinate on Chromosorb W (60–80 mesh, DMCS treated) packed in a 2·5 m × 4·5 mm i.d. stainless steel column. The first column was manually temperature programmed between 35° and 140° in an Aerograph 664. The presence of added carrier isobutyraldoxime in the samples was monitored by a thermal conductivity detector and the eluted compound trapped for liquid scintillation counting. The second column was used in an Aerograph 1520 fitted with an effluent stream splitter such that a minor portion of the eluted compounds passed to a flame ionization detector while the major portion passed through a Nuclear-Chicago 461 gas flow proportional radioactivity detector. A linear temperature programme from 35° to 190° at 4°/min was used with a carrier gas flow of 60 ml N_2/min. Radioactive peaks were trapped at the outlet of the proportional detector for further identification.

Identification of volatile labelled compounds. Both isobutyraldehyde and isobutyraldoxime were identified by preparing isobutyraldehyde 2,4-dinitrophenylhydrazone in dilute acid solution. After standing a few hours the mixtures were extracted with Et_2O and chromatographed on thin layers of silica gel alongside authentic markers. The solvents used were benzene, toluene, and *n*-butyl acetate–light petroleum (40–60°) (1:9, v/v). Isobutyraldehyde 2,4-dinitrophenylhydrazone was thereby distinguished from the corresponding hydrazones of acetaldehyde, acetone, butanone and methacrolein. A sample of the radioactive hydrazone derived from compound I was diluted with 200 mg of crystalline isobutyraldehyde 2,4-dinitrophenylhydrazone and recrystallized to constant specific activity.

Isobutyronitrile, isolated with ^{14}C label by GLC was identified by preparing *tert*-butylisobutyramide.[7] The sample was diluted with unlabelled isobutyronitrile (11 m-moles) and mixed with *tert*-butanol (20 m-moles) in HOAc (5 ml). The mixture was cooled prior to adding conc. H_2SO_4 (20 m-moles) and then held at 40° for 50 min. *tert*-Butylisobutyramide was precipitated by adding ice (20 g), filtered off and recrystallized from ethanol and water to constant specific activity. It sublimed at 110°.

Analysis of compound I labelled with D-*glucose*-U-^{14}C. D-Glucose-U-^{14}C (7 μmoles, 25 μc), L-valine (1 μmole) and DL-*o*-methylthreonine (10 μmoles) were administered to flax seedling shoots by the standard method for 7 hr. Two dimensional paper chromatography of a portion of the ethanolic extract and radioautography showed a range of labelled compounds including spots presumed to be linamarin and lotaustralin. A strong radioactive spot was observed in the position of compound I, corresponding exactly with that obtained in a parallel feeding treatment where L-valine-U-^{14}C was fed in place of D-glucose-U-^{14}C. Labelled compound I was absent in a control experiment with D-glucose-U-^{14}C when DL-*o*-methylthreonine was omitted.

The spot was eluted with H_2O and treated with 0·5 mg β-glucosidase in 0·5 ml phosphate buffer, (0·005 M, pH 6·0) for 15 hr at 25°. Upon subsequent paper chromatography in butanone–acetone–H_2O (15:5:3, v/v), propanol–H_2O (7:3, v/v), and EtOAc–pyridine–H_2O (2:1:2, v/v, top phase), half of the radioactivity had the same R_f as D-glucose and the remainder chromatographed with compound I.

Analysis of compound I labelled with L-*valine*-U-^{14}C. Compound I was prepared from L-valine-U-^{14}C in the presence of *o*-methylthreonine and isolated by paper chromatography. It was treated with β-glucosidase (2·5 mg) in sodium phosphate buffer (100 μl, pH 5·9, 0·05 M) for 20 hr at 40°. The hydrolysis products were extracted into Et_2O for GLC using the diethylene glycol succinate column. A small sample of I was also pyrolysed on a Nichrome wire heater in the inlet of the gas chromatograph. Radioactive isobutyraldehyde was the sole product detected from the acid treatment while radioactive isobutyraldoxime was the principal product of enzymic hydrolysis. Small amounts of radioactivity with retention times the same as isobutyraldehyde and isobutyronitrile were also present. Radioactive isobutyronitrile was the major labelled compound obtained by pyrolysis of I.

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[7] H. PLAUT and J. J. RITTER, *J. Am. Chem. Soc.* **73**, 4076 (1951).

Key Word Index—Linum usitatissimum; Linaceae; biosynthesis; linamarin; isobutyraldoxime *O*-β-glucoside.