Phytochemistry, 1972, Vol. 11, pp. 1047 to 1053. Pergamon Press. Printed in England.

2-HYDROXYALDOXIMES AS POSSIBLE PRECURSORS IN THE BIOSYNTHESIS OF CYANOGENIC GLUCOSIDES

B. A. TAPPER*, H. ZILG† and E. E. CONN

Department of Biochemistry and Biophysics, University of California, Davis, Calif. 95616, U.S.A.

(*Received* 25 *August* 1971)

Abstract—The possibility of 2-hydroxyaldoximes being alternative intermediates to nitriles in the biosynthesis of cyanogenic glucosides was investigated. 2-Hydroxyisobutyraldoxime-1,3-^{14}C and DL-2-hydroxyphenylacetaldoxime-2-^{3}H were administered to linen flax (*Linum usitatissimum* L.) and to cherry laurel (*Prunus laurocerasus* L.) respectively. The incorporation of these precursors into the corresponding glucosides was sufficient to indicate that they may be intermediates in the biosynthesis.

INTRODUCTION

STUDIES on the biosynthesis of cyanogenic glucosides have demonstrated that aldoximes may be intermediates between amino acids and the glucosides.[1-3] With linamarin (2-hydroxyisobutyronitrile-β-D-glucopyranoside) formation in linen flax (*Linum usitatissimum* L.), isobutyraldoxime was an effective precursor and its formation from L-valine was demonstrated. Isobutyronitrile and 2-hydroxyisobutyronitrile were also shown to be precursors and a biosynthetic sequence with these intermediates has been postulated,[4] as in Fig. 1. In this paper evidence for an alternative pathway involving 2-hydroxyaldoximes is examined

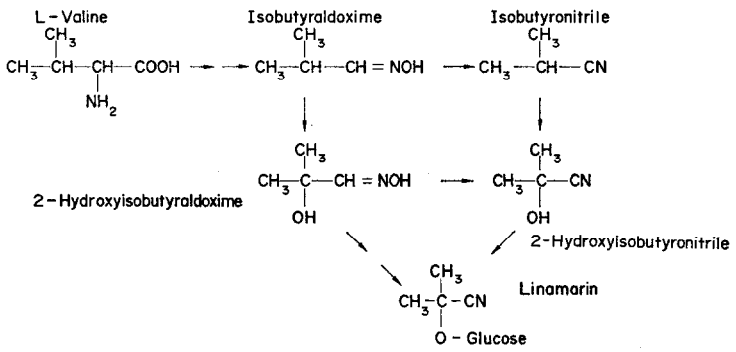

FIG. 1. PROPOSED PATHWAY OF LINAMARIN BIOSYNTHESIS. 2-HYDROXYISOBUTYRALDOXIME MAY BE AN ALTERNATIVE INTERMEDIATE TO ISOBUTYRONITRILE.

* Present address: Applied Biochemistry Division, D.S.I.R., Private Bag, Palmerston North, New Zealand.

† Present address: Biologisches Institut II, Universitat Freiburg, 78 Freiburg, Schaenzlestr 9–11, West Germany.

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[2] B. A. TAPPER and G. W. BUTLER, *Biochem. J.* **124**, 935 (1971).
[3] B. A. TAPPER and G. W. BUTLER, *Phytochem.* **11**, 1041 (1972).
[4] K. HAHLBROCK, B. A. TAPPER, G. W. BUTLER and E. E. CONN, *Arch. Biochem. Biophys.* **125**, 1013 (1968).

for the biosynthesis of linamarin in flax and prunasin (2-D-hydroxyphenylacetonitrile-β-D-glucopyranoside) in cherry laurel (*Prunus laurocerasus* L.).

RESULTS

Administration of 2-Hydroxyisobutyraldoxime,1,3-^{14}C to Linen Flax

The substantial incorporation of L-valine into linamarin in flax seedling shoots has been well established[5] and the incorporation of other compounds may be compared with it. The results of administering 2-hydroxyisobutyraldoxime-1,3-^{14}C and L-valine-U-^{14}C are given

TABLE 1. INCORPORATION OF ^{14}C-COMPOUNDS INTO LINAMARIN

	Compounds administered			Linamarin			
Expt.		Amount μmoles	S.A.*	Amount μmoles	S.A.*	% Precursor converted	Dilution of ^{14}C
1.	L-Valine-U-^{14}C	1·0	1050	13·3	22	34†	39†
	L-Valine-U-^{14}C in presence of 8 μmoles 2-hydroxyiso-butyraldoxime	1·0	1050	13·8	21	34†	40†
	2-Hydroxyiso-butyraldoxime-1,3-^{14}C	4·2	278	12·3	11	12	25
2.	L-Valine-U-^{14}C	1·0	2620	—	—	27†	—
	L-Valine-U-^{14}C in presence of 25 μmoles 2-hydroxyiso-butyraldoxime	1·0	2620	—	—	22†	—
	2-Hydroxyiso-butyraldoxime-1,3-^{14}C	22	19	—	—	3·0	—
	2-Hydroxyiso-butyraldoxime-1,3-^{14}C	1·5	278	—	—	12	—

* Specific activity in μc/mmole.
† Corrected for an assumed loss of -^{14}COOH from precursor.

in Table 1 where values observed for incorporation of radioactivity from the latter compound have been corrected by the factor of 5/4 to allow for the loss of carboxyl-^{14}C during the biosynthesis. The effect of unlabelled 2-hydroxyisobutyraldoxime on the incorporation of L-valine-U-^{14}C is also compared in this table.

The results show 2-hydroxyisobutyraldoxime was converted to linamarin but at a rate less than for L-valine when comparable amounts were administered. However, the incorporation is sufficient to suggest the 2-hydroxyisobutyraldoxime may be an alternative intermediate to isobutyronitrile which was incorporated into linamarin to about the same extent.[2,4] It is notable than when 8 μmoles of unlabelled 2-hydroxyisobutyraldoxime was administered with L-valine-U-^{14}C there was no reduction of incorporation of label into

[5] G. W. BUTLER and E. E. CONN, *J. Biol. Chem.* **234**, 1674 (1964).

linamarin and only when 25 μmoles of the oxime was so administered was there a small reduction of label incorporation.

Upon paper chromatography, extracts from the 2-hydroxyisobutyraldoxime-1,3-^{14}C treatment were shown to contain at least three major radioactive compounds. One spot with an R_f of 0·82 in butanone–acetone–water (15:5:3, v/v) ran identically with 2-hydroxyisobutyraldoxime and was probably the unconverted, administered compound. The linamarin spot with an R_f of 0·44 was followed by a spot of about three times the radioactivity with an R_f of 0·33. The compound giving this radioactive spot is designated compound II to distinguish it from compound I found previously by Tapper and Butler[3] when administering L-valine-U-^{14}C in the presence of various specific inhibitors of linamarin biosynthesis.

Compound II gave the most strongly labelled chromatographic spot when 4·2 μmoles or less of 2-hydroxyisobutyraldoxime-1,3-^{14}C was administered to 20 seedling shoots. The trimethylsilyl ether derivative of this compound was purified by GLC on an SE30 silicone column and the mass spectrum recorded. The two highest mass peaks were at *m/e* 625 and *m/e* 610 which are consistent with a structure of a penta-(trimethylsilyl) ether of a 2-hydroxyisobutyraldoxime monoglucoside.

Treatment of II with saturated 2,4-dinitrophenylhydrazine in 2% HCl gave a radioactive spot moving at the solvent front upon paper chromatography in butan-1-ol saturated with water. This spot was shown to be a mixture of methacrolein-2,4-dinitrophenylhydrazone and 2-hydroxyisobutyraldehyde-2,4-dinitrophenylhydrazone by TLC on silica gel in toluene. A similar mixture of hydrazones could be produced under corresponding conditions from 2-hydroxyisobutyraldoxime.

When II was treated with 2,4-dinitrophenylhydrazine in 2% HOAc at room temp. an additional radioactive spot was observed with an R_f of 0·78 using butan-1-ol saturated with water. This compound could be converted to the mixture of hydrazones of methacrolein and 2-hydroxyisobutyraldehyde by dil. HCl.

Administering D-glucose-U-^{14}C (10 μc) to flax shoots along with unlabelled 2-hydroxyisobutyraldoxime also yielded labelled compound II. Again a labelled derivative with the same chromatographic properties was formed with 2,4-dinitrophenylhydrazine in 2% HOAc and after additional treatment with 2% HCl the radioactivity was shown to be present in glucose by paper chromatography in butan-1-ol–pyridine–water (6:4:3, v/v). Accordingly the proposed structure of compound II is 2-(glucosyloxy)isobutyraldoxime. This structure would be expected to give a 2,4-dinitrophenylhydrazone under mildly acidic conditions and the glucose moiety would be hydrolysed off with stronger acid.

Readministration of compound II to flax shoots did not result in a detectable conversion to linamarin but 80% of the compound administered could be recovered from the shoot.

Volatile intermediates. In an attempt to induce accumulation of labelled intermediates, unlabelled 2-hydroxyisobutyraldoxime was administered to flax shoots along with L-valine-U-^{14}C. For comparison L-valine-U-^{14}C was also administered alone and with unlabelled isobutyronitrile. Each batch of shoots was extracted with 3 ml of cold diethyl ether by grinding with glass beads and 0·5 g sodium sulphate as described by Tapper and Butler[3] and samples taken for GLC on DC 550 and QF-1 silicone oil columns. Figure 2 gives the continuously recorded radioactivity in each treatment together with a typical separation trace and temperature program using the DC 550 silicone oil column.

The control treatment (Fig. 2A) showed a peak of radioactivity possibly containing

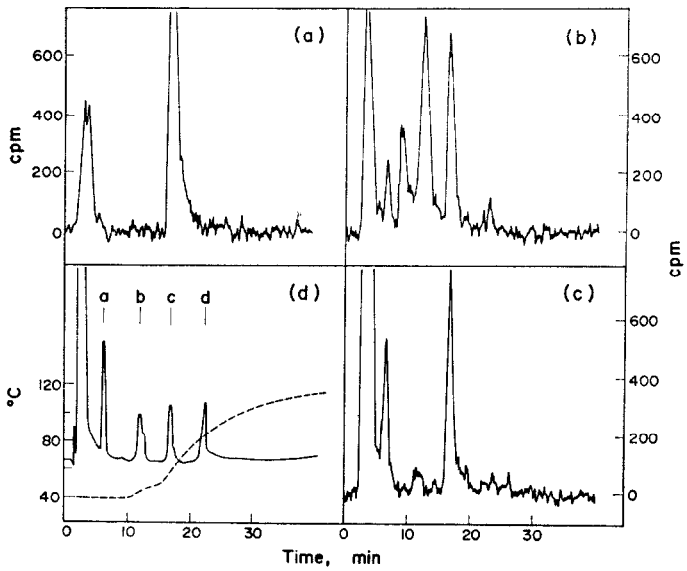

FIG. 2. GAS CHROMATOGRAPHY OF DIETHYL ETHER EXTRACTS OF FLAX SHOOTS TREATED WITH L-VALINE-U-^{14}C.

a: Radioactivity in control treatment. B: Radioactivity in the presence of 50 μmoles of 2-hydroxyisobutyraldoxime. C: Radioactivity in the presence of 60 μmoles of isobutyronitrile. D: Typical separation of isobutyronitrile (a), *syn* and *anti* isomers of isobutyraldoxime (b), 2-hydroxyisobutyronitrile (c) and 2-hydroxyisobutyraldoxime (d). The temperature Program is shown as a dotted line.

acetone eluting near the solvent front and another peak attributed to 2-hydroxyisobutyronitrile eluting at about 17 min. These peaks occurred in all treatments and were identified by collecting and converting to acetone 2,4-dinitrophenylhydrazone.

The treatment with 2-hydroxyisobutyraldoxime (50 μmoles, Fig. 2B) gave a significant peak of radioactive isobutyraldoxime (12 min) identified by treatment with acidic 2,4-dinitrophenylhydrazine and subsequent TLC. A small amount of radioactivity occurred with the elution time of 2-hydroxyisobutyraldoxime (23 min). Interestingly, two other small peaks of radioactivity occurred, one with the elution time of isobutyronitrile (6·5 min) and the other a little later (9 min). Neither peak was observed in the control.

When isobutyronitrile (60 μmoles, Fig. 2C) was administered the initial peak of radioactivity was increased and a small peak followed with the elution time of isobutyronitrile. In a similar experiment by Tapper and Butler[3] labelled isobutyronitrile was isolated and identified.

A portion of the extract of the 2-hydroxyisobutyraldoxime treatment was subjected to two-dimensional TLC on silica gel with benzene–methanol (1:1, v/v) and chloroform–ethanol–formic acid (2:1:1, v/v). The 2-hydroxyisobutyraldoxime spot was located by spraying with dilute ferric chloride. Autoradiography showed this spot to be radioactive though the total radioactivity in the oxime was very low (< 0·05% of the L-valine-U-^{14}C administered).

Administration of DL-2-*Hydroxyphenylacetaldoxime-2-*^{3}H *to Cherry Laurel*

The incoporation of the tritiated oxime into cyanogenic glucoside is compared in Table 2

TABLE 2. INCORPORATION OF LABELLED COMPOUNDS INTO CYANOGENIC GLUCOSIDE OF CHERRY LAUREL

Compound administered	Amount μmoles	S.A.†	Cyanogenic glucoside* HCN μmoles	S.A.†	% Precursor converted	Dilution of radioactivity
DL-2-Hydroxyphenylacetaldoxime-2-^{3}H‡	7·5	200	174	0·36	4·2	560
DL-2-Hydroxyphenylacetaldoxime-2-^{3}H	7·5	200	125	0·43	3·6	470
L-Phenylalanine-U-^{14}C	5	500	165	0·65	5·6§	600§
L-Phenylalanine-U-^{14}C with 25 μmoles DL-2-Hydroxyphenylacetaldoxime	5	500	197	0·68	6·9§	570§

* The cyanogenic glucoside was analysed by enzymic cleavage and reaction of products to give HCN and benzaldehyde semicarbazone. The specific activity (S.A.), and dilution of radioactivity were determined on the later compound while the % precursor converted is calculated from the specific activity and the amount of HCN measured.

† Specific activity in μc/mmole.

‡ This treatment was for 28 hr; the other treatments were for 15 hr.

§ Corrected by 9/7 for assumed loss of two carbons from L-phenylalanine-U-^{14}C.

to the well-established incorporation of L-phenylalanine-U-^{14}C into prunasin. As prunasin is difficult to separate from its diastereoisomer, sambunigrin (2-L-hydroxyphenylacetonitrile-β-D-glucopyranoside), it is not possible to state with certainty that prunasin is the only cyanogenic glucoside formed from the administered DL-2-hydroxyphenylacetaldoxime-2-^{3}H. The results show that the oxime was converted to cyanogenic glucoside at a rate similar to that of L-phenylalanine but considerably less than the reported incorporation rate of phenylacetonitrile-1-^{14}C which may be an alternative intermediate.[2,4] There was no effect on the incorporation of L-phenylalanine-U-^{14}C into prunasin when 25 μmoles of unlabelled DL-2-hydroxyphenylacetaldoxime was simultaneously administered. A depressed incorporation of radioactivity would be expected if the oxime was an intermediate. Three major radioactive bands were observed after paper chromatography of the extract from the DL-2-hydroxyphenylacetaldoxime treatment. One of these with a high R_f in butanone–acetic acid–water (200:10:25, v/v) was attributed to the unconverted administered compound. The single labelled cyanogenic glucoside spot had an R_f of 0·67 and was only partially separated from the third radioactive spot with an R_f of 0·50. This compound, III, was not identified although its chromatographic properties indicated it may have been a glucoside analogous to compound II observed from flax shoots. Compound III did not yield benzaldehyde upon treatment with β-glucosidase.

DISCUSSION

The experiments described were performed to test whether 2-hydroxyaldoximes could be precursors of corresponding cyanogenic glucosides and possible alternatives to nitriles as intermediates in the biosynthetic pathway. The incorporation rates observed for both 2-hydroxyisobutyraldoxime and DL-2-hydroxyphenylacetaldoxime into cyanogenic glucosides was sufficient to indicate a possible intermediate role although the results were no more convincing than corresponding experiments with the alternative nitriles.[2,4]

Competition experiments with the 2-hydroxyaldoximes were essentially negative but the positive competition effect observed with isobutyronitrile in flax[3] is at least partially due to

the inhibitory action on the final glucosylation step.[6] Attempts to trap the two alternative intermediates in the flax system were successful in both cases, although more radioactivity was accumulated in the nitrile treatment. However, positive results with competition and trapping experiments can only be expected if exogenously administered compounds exchange freely with the corresponding pools and failing proof of this being so these types of experiment can be misleading.

The data presented in this paper do not allow a decision on whether nitriles, 2-hydroxyaldoximes or both are intermediates in the biosynthesis of cyanogenic glucosides. That problem will best be answered at the enzymic level.

EXPERIMENTAL

L-Valine-U-^{14}C, D-glucose-U-^{14}C and L-phenylalanine-U-^{14}C were obtained from the New England Nuclear Corp. Isobutene-1,3-^{14}C with approximately 50% of the total label at C-1 was obtained from Mallinckrodt Nuclear and used to prepare 2-hydroxyisobutyraldoxime-1,3-^{14}C by the method of Nenz and Ribaldone.[7] Sodium borohydride-^{3}H in tetrahydrofuran solution was obtained from Schwarz Bioresearch Inc., and used to prepare DL-2-hydroxyphenylacetaldoxime by an adaption of the method of Samne and Freon.[8]

2-*Hydroxyisobutyraldoxime*-1,3-^{14}C. Isobutene-1,3-^{14}C (2 mmoles, approximately 0·5 mc) was trapped in pentane (0·2 ml) at −15° and treated at that temperature with nitrosylsulphuric acid (2 mmoles) dissolved in 80% (w/w) H_2SO_4 (0·72 g). The mixture was shaken for 90 min before removing the pentane and unreacted isobutene by evaporation under reduced pressure at 0°. The residue together with ice (3 g) was neutralized to pH 7·8 with NH_4OH and extracted with Et_2O (×3). The ether extract, after drying and concentrating, was purified by GLC using a SE30 silicone gum column and TLC on silica gel G using benzene–MeOH (9:1, v/v). The 2-hydroxyisobutyraldoxime-1,3-^{14}C, with a yield of about 10%, was identified by comparison of its chromatographic properties with an unlabelled sample prepared on a larger scale by the same procedure. The unlabelled compound was purified by distillation at 68° to 71° at 0·3 mm Hg pressure, n_d^{20} = 1·4620 (lit., 1·4628, 1·4628,[7]). The NMR spectrum in $CDCl_3$ showed two sharp signals—one at 1·38 ppm (six methyl protons) and the other at 7·52 ppm (proton at the oximino carbon)—and very broad signals for the hydroxyl protons.[9] A volatile compound, tentatively identified as 1-*tert*-butoxyimino-2-hydroxy-2-methylpropane by NMR and mass spectrometry, was observed as a by-product.

DL-2-*Hydroxyphenylacetaldoxime*-2-^{3}H. Phenylglyoxaldoxime (0·6 mmoles) was added to sodium borohydride-^{3}H (0·2 mmoles) dissolved in tetrahydrofuran (1 ml) and isopropanol (1 ml). The mixture was shaken for 30 min at 25° and then 2 hr at 40° before adding H_2O (1 ml) and extracting with Et_2O (×4). The combined extract was concentrated and treated with four successive portions of H_2O (1 ml) which was evaporated off each time by a stream of N_2 to remove freely exchangeable tritium from the product.

The DL-2-hydroxyphenylacetaldoxime-2-^{3}H was further purified on a silica gel column using benzene–MeOH (20:3, v/v) as eluent. The fractions containing the product were concentrated and the residue crystallized from toluene. TLC showed the product was radiochemically homogeneous. The radioactivity in the product was not exchanged with water when held at 100° for 20 min or when treated with dil. HOAc or NH_4OH.

The labelled compound was identified by comparison with unlabelled DL-2-hydroxyphenylacetaldoxime prepared by the same method on a larger scale. M.p. 89–90° (lit. m.p. 93–94°[8]).

Plant material. Linen flax seedling shoots and young cherry laurel shoots were obtained and compounds administered to them as previously described.[2] The flax shoots were used in batches of twenty and the cherry laurel in batches of three (about 2·5 g fresh wt.).

Extraction and analysis. The extraction and analysis generally followed described procedures.[2,3] Ethanolic extracts were made for non-volatile compounds and concentrated for paper chromatography. Extracts of flax by small volumes of Et_2O[3] were used for GLC on columns of 14% (w/w) QF-1 fluorosilicone on Anakrom AB (60/80 mesh, Analabs, Inc.) packed in 3 m × 4·5 mm i.d. stainless steel and 10% DC 550 silicone on Chromasorb W (70/80 mesh, DMCS treated) packed in a 1·6 m × 4·5 mm i.d. stainless steel tube. An Aerograph gas chromatograph, Model A-90-P, was used with temperature programming from about 35° to over 100°. Both columns resolved isobutyronitrile, isobutyraldoxime, and 2-hydroxyisobutyraldoxime from each other and from the solvent peak. 2-Hydroxyisobutyronitrile was also separated on the DC 550

[6] K. HAHLBROCK and E. E. CONN, Unpublished results.
[7] A. NENZ and G. RIBALDONE, *Chim. Ind.* (*Milan*) **49**, 43 (1967).
[8] S. SAMNE and P. FREON, *C.R. Acad. Sci. Paris* **254**, 1643 (1962).
[9] E. PERROTTI, M. LANZONI, N. PALADINO and M. DE MALDE, *Ann. Chim.* (*Rome*) **56**, 1379 (1966).

silicone column but apparently decomposed on the QF-1 column. The effluent from the gas chromatograph was passed through a Nuclear-Chicago 461 proportional gas flow detector.

The trimethylsilyl ether of compound II was prepared by the method of Sweeley *et al.*[10] and purified by GLC with a 3% w/w SE30 silicone gum on Chromosorb W column (2 m × 4·5 mm i.d.) at 190°.

The incorporation of labelled compounds into the cyanogenic glucoside from cherry laurel was measured by treating portions of the extracts with β-glucosidase in flasks with separate wells containing semicarbazide hydrochloride solution to trap benzaldehyde and dilute NaOH to trap HCN.[2] This method did not distinguish between prunasin and its diastereoisomer, sambunigrin.

[10] C. C. SWEELEY, R. BENTLEY, M. MAKITA and W. W. WELLS, *J. Am. Chem. Soc.* **85**, 2497 (1963).

Key Word Index—Linum usitatissimum; Linaceae; *Prunus laurocerasus*; Rosaceae; biosynthesis; cyanogenic glucosides; 2-hydroxyaldoximes.