Stereocchemical Aspects of Lotaustralin Biosynthesis*

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Harald Zilg‡ and Eric E. Conn
From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616

SUMMARY

The methyl linamarin that occurs naturally in linen flax (Linum usitatissimum L.) seedlings was shown to have the R configuration, i.e. it is identical with (R)-lotaustralin, (R)-2-hydroxy-2-methylbutyryonitrile-β-D-glucopyranoside. However, when a mixture of the two enantiomers (R,S)-2-hydroxy-2-methylbutyryonitrile, was administered to excised flax shoots, both (R)-lotaustralin and its epimer (S)-epi-lotaustralin ((S)-2-hydroxy-2-methylbutyryonitrile-β-D-glucopyranoside) were formed. Similarly, both (R)-lotaustralin and (S)-epi-lotaustralin were formed when (R,S)-2-hydroxy-2-methylbutyryonitrile served as substrate for the UDP-glucose:ketone cyanohydrin-β-d-glucosyltransferase of flax. These results demonstrate that the glucosyltransferase does not distinguish either in vitro or in vivo between the two enantiomeric forms of this glucose acceptor. Since, however, only (R)-lotaustralin occurs in the intact plant, the glucosyltransferase must be presented with only the R enantiomer as a substrate. This form is produced from l-isoleucine ((3S)-l-isoleucine) due to retention of the S configuration at carbon atom 3. When (3R)-l-isoleucine (threo-l-isoleucine) was administered to flax shoots, (S)-epi-lotaustralin was formed due to retention of configuration at carbon atom 3 of the amino acid.

The cyanogenic glucoside linamarin (I) (Fig. 1) occurs with its higher homologue lotaustralin in a number of higher plants (1). The aglycones of these two glucosides are formed from the amino acids valine and isoleucine (2), respectively. The biosynthetic pathway which is involved has been described (3).

In contrast to linamarin, the aglycone of lotaustralin, 2-hydroxy-2-methylbutyronitrile, contains a chiral center; therefore, two epimeric β-glucosides could, in theory, occur in nature. These two glucosides, which have been termed methyl linamarins, having the R configuration at the glucosidic carbon has been designated as (R)-lotaustralin (II) and is the glucoside which occurs naturally in Trifolium repens (4). The epimer, which to date has not been found as a naturally occurring glucoside, was designated as (S)-epi-lotaustralin (III).

The work of Bissett et al. (4) has made it possible to identify the methyl linamarin that occurs naturally in linen flax as well as to examine the product of the action of the UDP-glucose:ketone cyanohydrin-β-glucosyl transferase from flax (5) that catalyzes the glucosylation of 2-hydroxy-2-methylbutyryonitrile. In addition, it has been possible to show that the configuration of the methyl linamarin that is found in flax is determined by the configuration of the β-carbon of the amino acid that serves as its precursor.

EXPERIMENTAL PROCEDURES

Materials—D-[U-14C]Glucose was obtained from New England Nuclear Corporation while potassium [14C]cyanide was obtained from Schwarz Bioreserach Company and used to prepare the racemic mixtures of the R and S enantiomers of 2-hydroxy-2-methylbutyryl [14C]nitrite, 2-hydroxy-2-ethylbutyryl [14C]nitrite, and 1-hydroxy-cyclopentane[14C]carbonitrile by the method of Tapper et al. (6). (R) Lotaustralin tetraacetate and (S)-epi-lotaustralin tetracetate were synthesized and separated according to the method of Bissett et al. (4). L-Cyclopentanone-glycine and D-2-cyclohexanone-1-glycine were gifts of Professor W. Shive, University of Texas, Austin. Samples of (R)-lotaustralin, (S)-epi-lotaustralin and deidaclin were kindly provided by Dr. M. G. Ettlinger and Dr. R. C. Clapp, Army Laboratories, Natick, Massachusetts. (3S)-L-Isoleucine or erythro-l-isoleucine, the amino acid derived from proteins, was purchased from Mann Research Laboratories; in this paper, its trivial name, l-isoleucine, is used. (3R)-l-Isoleucine or threo-l-isoleucine, which has the opposite or R configuration at carbon 3, was also purchased from Mann Research Laboratories; this sample was shown to contain 20% l-isoleucine when subjected to an amino acid analysis according to the method of Moore et al. (7). D-2-Amino-3-ethylvaleric acid was prepared by synthesis of the hydantoin (8) starting from 2-ethylbutyraldehyde. Hydrolysis of the hydantoin yielded the crude amino acid which was purified by precipitation from methanolic solution with pyridine followed by crystallization from water. Thin layer chromatography on silica gel with chloroform-methanol-17% aqueous ammonia (2:1:1 v/v/v) (9) showed one single ninhydrin-positive spot at Rf 0.49 (valine, 0.38; isoleucine, 0.43). The dinitrophenyl derivative (10) exhibited a melting point of 159°. The mass spectrum showed the characteristic fragmentation pattern of a dinitrophenyl-amino acid (11) with a M+ peak at m/e 311, the base peak at m/e 296 derived by loss of COOH from M+, and other peaks produced by fragmentation of the side chain or the dinitrophenyl group.

Administration of Compounds to Flax Seedlings—Flax seed (Linum usitatissimum L., obtained from the California Crop Improvement Office, University of California, Davis) were germinated and seedlings grown 3 days in the dark and then for 20
The sheets were then counted for radioactivity and the glucosides visualized by spraying with cw-naphthol and sulfuric acid and subsequent heating at 105º for 5 min.

Stereochemistry of Glucosylation Step—When, in an earlier study (5), 2-hydroxy-2-methylbutyronitrile was examined as a substrate for the UDP glucose:ketone cyanohydrin-β-glucosyltransferase of flax, it was readily glucosylated as was both its lower homologue, 2-hydroxyisobutyril cyanohydrin and its next higher homologue, 2-hydroxy-2-ethylbutyronitrile. Although the 2-hydroxy-2-methylbutyronitrile employed in that study existed as the racemic mixture of R and S enantiomers, no method was available to determine whether one or both of the enantiomers served as a substrate for glucosylation. The studies of Bissett et al. (4) made it possible to examine this matter both in the intact plant and with the partially purified flax glucosyltransferase.

(R,S)-2-Hydroxy-2-methylbutyronitrile[14C]nitrile (0.5 μmol; 1.7 × 10⁶ dpm) was administered to 10 excised flax shoots in a closed vessel and allowed to metabolize in the light for 6 hours. The shoots were then extracted in boiling 80% ethanol and chromatographed first in Solvent System a and then in Solvent System b. The main radioactive product in the ethanol-soluble extract, which possessed the same Rf as methyl linamarin in the two solvent systems, was then eluted from the paper by the second solvent system, acetylated, and examined by thin layer chromatography in the BN chamber. Analysis of the radioactivity on the plate (Fig. 2) showed that 60% of the newly synthesized methyl linamarin consisted of (R)-lotaustralin and the balance (40%) was (S)-epi-lotaustralin. The total incorporation of radioactivity in the two epimers corresponded to 26% of the nitrile originally administered to the shoots.

Since the glucosylation reaction in the intact shoots appeared to proceed with both enantiomers of the aglycone of lotaustralin, the action of the glucosyltransferase in vitro was then examined. The UDP-glucose:ketone cyanohydrin-β-glucosyltransferase of flax was purified through Step 3 (ammonium sulfate fractionation) of the purification procedure (5). Forty units (0.5 mg of protein) of the enzyme were incubated with 2 μmoles of (R,S)-2-hydroxy-2-methylbutyronitrile[14C]nitrile (6.65 × 10⁶ dpm) in the standard assay (5). After the radioactive product had been

Mass spectra were recorded with a Varian M 66 mass spectrometer.
obtained by paper chromatography, in two solvent systems, of the original plant extract was acetylated. An aliquot (10%) of the acetylated mixture was then spotted on prepared silica gel sheets, chromatographed in the BN chamber, scanned, and autoradiographed. The chromatography tracing is matched with the intact seedlings.

Administration of Aliphatic Amino Acids to Flax Seedlings—Since only (R)-lotaustralin occurs in flax, the glucosyltransferase must be present in vivo with only one of the enantiomers of 2-hydroxy-2-methylbutyronitrile as a substrate for glucosylation. Moreover, this fact must be determined by the S configuration of carbon atom 3 of the L-isoleucine that serves as a precursor. If (3R)-L-isoleucine with the opposite configuration of that carbon atom (Fig. 1) were fed, the cyanogen produced by the flax plant should be the unnatural or (S)-epi-lotaustralin.

To test this hypothesis, we examined the nature of the glucosides formed when either L-isoleucine or (3R)-L-isoleucine was administered to flax shoots. Since (3R)-L-isoleucine labeled with ^{14}C was not readily available, advantage was taken of the fact (14, 15) that the sugar moieties of the cyanogenic glucosides became labeled when ^{14}C glucose is administered either separately or simultaneously with their amino acid precursor.

Excised flax shoots were pretreated with water or with solutions of 50 mM L-isoleucine or (3R)-L-isoleucine (Table I). After 2 hours, the shoots were then transferred to new solutions containing in addition 2.8 μCi of d-[U-^{14}C] glucose. After 9 hours of metabolism in the light, the shoots were extracted in boiling 80% ethanol and the radioactive glucosides purified on paper in Solvent Systems a and b, the suspension centrifuged, the solvent evaporated, and the residue solubilized and counted. The radioactive products corresponding to (R)-lotaustralin tetraacetate contained 13,250 dpm; that corresponding to (S)-epi-lotaustralin tetraacetate, 8,850 dpm. When corrected for aliquot size, the former represented 8.5 X 10^6 dpm, the latter 1.77 X 10^6 dpm. The sum of these amounts to 26% of the ^{14}C-labeled nitrile originally administered to the intact seedlings.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Amino acid administered</th>
<th>Per cent incorporation into cyanogenic glucosides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Linamarin plus methyl linamarin</td>
<td>Linamarin</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>4.61</td>
</tr>
<tr>
<td>2</td>
<td>L-isoleucine</td>
<td>4.25</td>
</tr>
<tr>
<td>3</td>
<td>(3R)-L-s-Isoleucine</td>
<td>4.67</td>
</tr>
</tbody>
</table>

TABLE II

Paper chromatographic and gas chromatographic separation of cyanogenic glucosides

<table>
<thead>
<tr>
<th>Cyanogenic glucoside</th>
<th>Re values by paper chromatography</th>
<th>Gas-liquid chromatography (Retention volume of the trimethylsilyl derivatives)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linamarin</td>
<td>0.45</td>
<td>590</td>
</tr>
<tr>
<td>Lotaustralin</td>
<td>0.55</td>
<td>710</td>
</tr>
<tr>
<td>2-Hydroxy-2-ethylbutyronitrile-β-δ-glucoside</td>
<td>0.68</td>
<td>810</td>
</tr>
<tr>
<td>1-Hydroxy-cyclopentane carbonitrile-β-δ-glucoside</td>
<td>0.57</td>
<td>1100</td>
</tr>
<tr>
<td>Deidaclin</td>
<td>0.57</td>
<td>1300 (decomposition)</td>
</tr>
</tbody>
</table>

*On a 3% SE-30 column. For details see “Experimental Procedures.”
cially available (3R)-

**Administration of Alcyclic Amino Acids to Flax Seedlings**—Tantisewicz et al. (16) have recently described the occurrence of a cyanogenic glycoside, deidacalin, which could, as Clapp et al. (17) point out, be derived from the amino acid 2-cyclopentene-1-glycine. In view of the capacity of the flax glucosyltransferase to accept a variety of cyanohydrins as substrate (5), we examined the ability of excised flax shoots to synthesize alcyclic glycosides of the cyclopentane-glycine type.

Encouraging results were obtained when nL-2-hydroxy-2-ethyl-butyronitrile and 1-hydroxycyclopentane-1-carbonitrile were administered to flax shoots. On administration of the former and analysis of the alcohol extract of the plants, one main radioactive product was obtained in a yield of 57%. This was tentatively identified as 2-hydroxy-2-ethylbutyronitrile-glucoside based on its behavior (Table II) as a higher homologue of lotaustralin on paper chromatography in Solvent Systems c and d and in gas-liquid chromatography.

Administration of the cyanohydrins of cyclopentanone also resulted in one major radioactive product (yield of 32%) which could not be distinguished from lotaustralin by paper chromatography. Therefore, the compound was examined, after silylation, in a combination gas chromatograph-mass spectrometer.

The fact that these cyanohydrins can be glucosylated in flax shoots is in agreement with the known broad specificity of the flax glucosyltransferase (5). Further, these results suggested that the flax plants might be able to synthesize those glucosides as well as deidacalin (1-cyano-1-hydroxycyclopentene-β-glucoside) when given the appropriate amino acid as precursors. Accordingly, nL-2-amino-3-ethylvaleric acid, nL-cyclopentane-1-glycine, and nL-2-cyclopentene-1-glycine were tested as precursors in an experiment similar to that described in Table I. The amino acids (12.5 μmoles) were administered in 0.25 ml of water to 10 flax shoots and after 1 hour of illumination, 0.15 μ mole (2 μCi) of [U-14C]glucose was added. After a 7-hour period of metabolism, the shoots were extracted and examined for the presence of the corresponding glucoside. Procedures involving initial purification on paper, silylation, and examination with gas-liquid chromatography-mass spectrometer failed to indicate the presence of any of the three glucosides that might be expected. However, the presence of the amino acids did inhibit the synthesis of linamarin and (R)-lotaustralin in amounts varying from 40 to 80%.

**DISCUSSION**

Several lines of evidence have indicated that a single set of enzymes is responsible for the conversion of valine and isoleucine to linamarin and (R)-lotaustralin in cyanogenic plants. Thus, genetic studies of cyanogenesis in *Trifolium repens* (19) and the effects of an inhibitor, O-methylthreonine, on the biosynthesis of linamarin and (R)-lotaustralin in flax (14) suggest a single set of biosynthetic enzymes. Moreover, competition experiments (20) with the enzyme which catalyzes the final step in linamarin biosynthesis indicates that it is also responsible for the final step in lotaustralin formation. To these observations may be added the principal finding reported in this paper, namely that the set of enzymes which synthesizes the cyanogenic glucosides of flax tolerates in vivo an exchange of methyl and ethyl groups at carbon atom 3 of the precursor amino acid and its subsequent metabolites. Moreover, the glucosyltransferase of flax uses both enantiomers of 2-hydroxy-2-methylbutyronitrile as substrates, thereby accepting interchangeably methyl and ethyl groups.

The formation of (S)-epi-lotaustralin from (3R)-L-isoleucine clearly establishes that the configuration of carbon atom 3 in isoleucine determines the configuration of the aglucone end product. Thus, the S configuration of that carbon atom in t-isoleucine is retained as (R)-lotaustralin is formed. The step in the biosynthetic pathway which directly involves carbon atom 3 is the one in which oxygen is introduced. The participation of molecular O2 in that reaction has recently been demonstrated (18). Since retention of configuration has been observed for a number of hydroxylations (21) catalyzed by mixed function oxidases, the retention of configuration observed in these experiments with L-isoleucine and (3R)-L-isoleucine provides further support for the involvement of such an enzyme in the biosynthesis of cyanogenic glucosides.

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