Biosynthesis of Cyanogenic Glycosides in Butterflies and Moths: Incorporation of Valine and Isoleucine into Linamarin and Lotaustralin by *Zygaena* and *Heliconius* Species (Lepidoptera)

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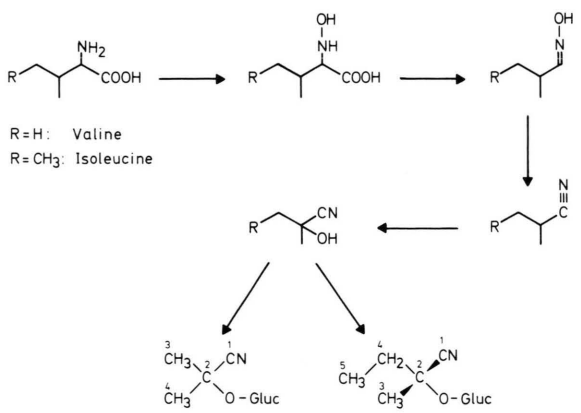
C-13 NMR data for linamarin and lotaustralin, obtained after feeding of either *Zygaena filipendulae* or *Heliconius melpomone* (Lepidoptera) with C-13 enriched valine and isoleucine respectively, indicate that intact units of amino acid with loss of the carboxyl group are incorporated during the biosynthesis of these cyanogenic glycosides.

Introduction

The moth *Zygaena filipendulae* and several related species (Zygaenidae: Lepidoptera), as well as the butterfly *Heliconius melpomone* and several related species (Nymphalidae: Lepidoptera), have been shown to contain the cyanogenic glucosides linamarin (2-β-D-glucopyranosyloxy-2-methylpropionitrile, **1**) and lotaustralin (2-β-D-glucopyranosyloxy-2*R*-methyl-butyronitrile, **2**) in all life stages [1–4]. Incorporation experiments with carbon-14 labelled valine and isoleucine, known to be precursors for linamarin and lotaustralin respectively from plant biosynthesis [5], resulted in incorporation rates of 2–6% when fed to larvae of *Zygaena filipendulae* and *Heliconius melpomone* [4, 6]. In plant biosynthesis valine and isoleucine are incorporated into linamarin and lotaustralin *via* the corresponding N-hydroxyamino acid, the aldoxime, the nitrile and the α-hydroxynitrile as shown in scheme 1; thus only the carboxyle carbon of the amino acid precursor is lost during plant biosynthesis whereas the residual carbon skeleton is totally incorporated into the cyanoglucosides [5]. The question arises whether during biosynthesis by these Lepidoptera both amino acids are incorporated in the same manner. Therefore, U-^{13}C labelled valine and U-^{13}C labelled isoleucine have been fed to the larvae of *Zygaena filipendulae* and *Heliconius melpomone* together with their normal food source and the localization of the carbon-13 label within the cyanoglucosides has been studied by carbon-13-NMR spectroscopy.



Scheme 1. Linamarin (1) Lotaustralin (2)

Experimental

L-[U-^{13}C]valine and L-[U-^{13}C]isoleucine, 85% enriched with carbon-13, were supplied by CEA

Request for reprints to Prof. Dr. A. Nahrstedt.

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(Gif sur Yvette, France). 20 mg of each amino acid was administered to sets of penultimate and final instar larvae in small portions on their natural food source; 32 *Z. filipendulae* larvae on *Lotus corniculatus* and 40 *H. melpomone* larvae on *Passiflora coerulea*. A solution of 20 mg of each amino acid was dissolved in 0.1 ml HCl (25%), 0.1 ml H_2O and 3.8 ml MeOH. Small amounts of this solution were dropped on to leaves which were accepted by the larvae after evaporation of the solvents.

After the feeding period the larvae were deep frozen with liquid nitrogen, then minced and freeze dried yielding 605 mg of *H. melpomone* containing ca. 5 mg linamarin and 3 mg lotaustralin, and 1718 mg of *Z. filipendulae* containing ca. 70 mg linamarin and 50 mg lotaustralin (for quantitative evaluation see [1, 2]). The dry material was extracted as described [1, 2] and the concentrated extract was chromatographed on a silica gel column (2 × 25 cm) using CH_2Cl_2/MeOH 80:20 as the solvent. The cyanide positive fractions [1] were collected and separated into linamarin and lotaustralin by HPLC on RP-18 (1.6 × 25 cm) using H_2O/MeCN 95:5 as the solvent (flow: 5 ml/min, RI detector; t_R linamarin: 19 min, t_R lotaustralin 47 min). Both glucosides were collected separately, evaporated to dryness and prepared for NMR spectroscopy by dissolving in 0.5 ml D_2O. The yield of both glucosides was about 50–60% of the amount in the larvae.

Proton decoupled ^{13}C-spectra were recorded at 100 MHz on a Bruker WM 400 NMR spectrometer using sweep widths of 25 000 Hz, pulse repetition rates of 0.655 s, a pulse angle of 23° and data lengths of 32 K. Fourier transforms of 128 K were performed with zero filling to improve line shape and precision. Spectral simulations were performed using a modified LAOCOON III program on a DEC-2020 computer. The experimental spectra shown in Figs. 1 to 3 have had Gaussian multiplication performed on their free induction decays in order to improve resolution, except in those cases indicated.

Results and Discussion

In the proton decoupled C-13 spectrum of linamarin isolated from the larvae of both species only the aglycone moiety shows additional signals from carbon-carbon couplings [7]. Thus the coupling between C-1 and C-2 of 60 Hz appears on both signals and is characteristic of such bonds [8]. Similarly the coupling between C-2 and C-3, and C-2 and C-4 of 39 Hz is characteristic of this bond type [7]. As expected only one bond couplings are observed as couplings over two bonds are known to be small, less than 3 Hz [9], for this system and hence are unobservable with the linewidths of 3.8 Hz found here. The splitting arising from the one bond coupling, of course, appears on both carbon signals and thus the signal for C-2 appears as a complex pattern the appearance of which depends on the number of enriched carbon atoms present per aglycone moiety.

If only one carbon per aglycone moiety is enriched then the signal of C-2 would appear as an intense singlet with signals arising from carbon-carbon coupling appearing almost symmetrically around the main signal with the outer lines being 60 Hz apart and the inner lines 39 Hz apart. The intensities of these satellite lines are small as doubly enriched molecules only have the natural abundance of 1.1% no matter what the amount of single carbon enrichment. Thus if each carbon was equally enriched then each outer line has an intensity of 0.55% and each inner line 1.1% (i.e. two couplings of 39 Hz) of the central singlet. Clearly this is not compatible with the observed spectrum for C-2 (Fig. 1). Similar considerations can be used to show that the experimental spectrum is not compatible with the presence of a *single* aglycone species consisting of two, three or four enriched carbon atoms per molecule.

However, as only 85% universally labelled valine was used in the feeding experiment this will also correspond to the maximum enrichment of the aglycone moiety. Thus for randomly labelled 85% C-13 enriched aglycone the proportions of each species involving C-2 are as shown in Table I. A composite theoretical spectrum, taking into account the fractional content of each species and their isotope shifts, is shown in Fig. 1, together with the experimental spectra of C-2 of linamarin from *Heliconius* and *Zygaena* larvae.

The excellent correspondence between the theoretical and experimental spectra with regard to line positions and relative intensities is direct evidence that only intact molecules of valine with loss of the carboxyl carbon were incorporated into the aglycone of linamarin. Any break down of valine during

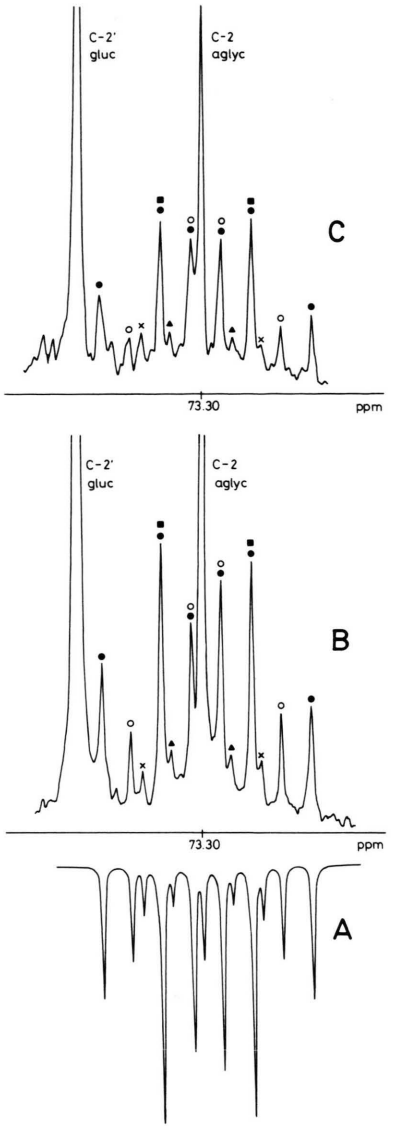

Fig. 1. Comparison of the experimental proton-decoupled C-13 NMR spectra of C-2 of linamarin from *Zygaena* (B) and *Heliconius* (C) with that calculated (A) for 85% universally enriched aglycone moiety, containing species **a** (●), **b** (○), **c** (×), **d** (■) and **e** (▲).

the incorporation process would lead to an increase in the proportion of species *b* to *f* and hence to the corresponding increase in intensity of their signals in the spectra. This is clearly not the case.

The degree of incorporation of labelled valine can be calculated from the signals heights in the multiplets for each peripheral carbon atom of the aglycone moiety by taking into account the various species present (Table I) that arise from the use of 85% universally labelled amino acid.

Thus, within the experimental error all lines of the aglycone moiety have been enriched to the same

Table I. Proportions of each species involving C-2 of 85% universally C-13 enriched aglycone moiety of linamarin. For lotaustralin the similarity of the couplings to C-2 of C-3 and C-4 does not allow distinction between species involving labelled C-3 or C-4 with labelled C-2. For example species **b** in fact includes both Gluc–O–C*(C*N), $(C^*H_3)CH_2CH_3$ and Gluc–O–C*(C*N) (CH_3) $C^*H_2CH_3$.

Species	No. of labels	Fractional content
a Gluc–O–C(*CN)(*CH_3)(*CH_3)	4	0.5220
b Gluc–O–C(*CN)(*CH_3)(CH_3)	3	0.1842
c Gluc–O–C(CN)(*CH_3)(*CH_3)	3	0.0921
d Gluc–O–C(*CN)(CH_3)(CH_3)	2	0.0163
e Gluc–O–C(CN)(*CH_3)(CH_3)	2	0.0325
f Gluc–O–C(CN)(CH_3)(CH_3)	1	0.0029
g Gluc–O–C(CN)(CH_3)(CH_3) *	0	0.1500

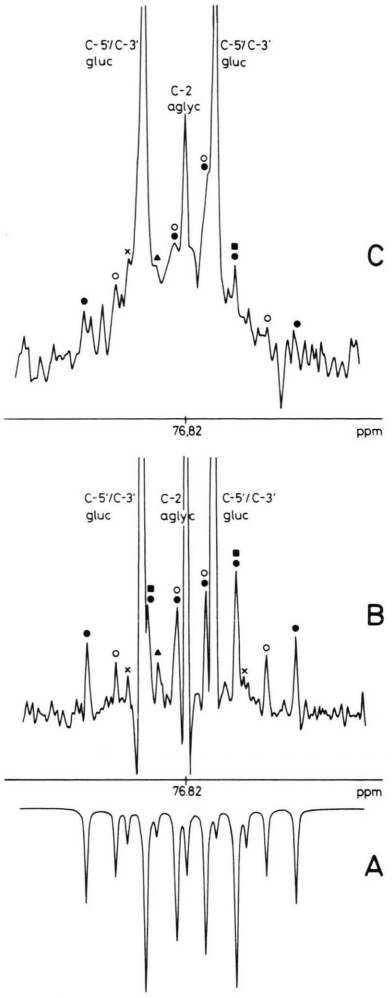

Fig. 2. Comparison of the experimental proton-decoupled C-13 NMR spectra of C-2 of lotaustralin from *Zygaena* (B) and *Heliconius* (C, without Gaussian multiplication), with that calculated (A) for an aglycone moiety containing all five carbon atoms enriched to 85%. Assignments see Fig. 1.

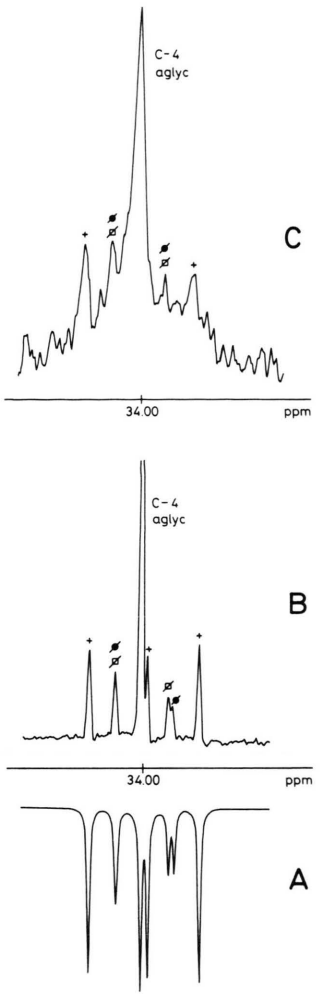

Fig. 3. Comparison of the experimental proton-decoupled C-13 NMR spectra of C-4 of lotaustralin from *Zygaena* (B) and *Heliconius* (C, without Gaussian multiplication) with that calculated (A) for an aglycone moiety containing all five carbons enriched to 85%. For C-4 the species are

Gluc–O–C*(CN)(CH_3)C*H_2CH_3 (●),
Gluc–O–C*(CN)(CH_3)C*H_2C*H_3 (+) and
Gluc–O–C(CN)(CH_3)C*H_2C*H_3 (⊘).

extent of $3.1 \pm 0.2\%$ for linamarin from larvae of *Z. filipendulae* and of $2.4 \pm 0.2\%$ for linamarin from larvae of *H. melpomone* while those of the glucose moiety did not show any evidence of double or multiple labelling.

The same considerations apply to the spectrum of lotaustralin obtained from the same larvae. As the magnitude of the couplings of C-2 to C-3 and C-4 are very similar the form of the theoretical spectrum for C-2 is similar to that for linamarin. Thus for lotaustralin the various species involving C-2 in Table I are also present with no distinction being possible between the labelled methyl group C-3 or the ethyl group with a label at C-4. Although the signal for C-2 of the aglycone moiety is overlapped by those for C-5′ and C-3′ of the glucose moiety (Fig. 2) the signal shows the same general pattern as that for linamarin (Fig. 2) at least for C-2 of lot-

Table II. C-13 chemical shifts, carbon-carbon constants and isotope shifts for linamarin and lotaustralin from *Z. filipendulae* (similar results were obtained for both glucosides from *H. melpomone*).

Chemical shifts [ppm] of unenriched species:

Glucose moiety

	C1′	C2′	C3′[a]	C4′	C5′[a]	C6′
Linamarin	99.92	73.86	76.55	70.52	77.10	61.66
Lotaustralin	99.60	73.87	76.64	70.54	77.11	61.64

Aglycone moiety

	C1	C2[b]	C3	C4	C5
Linamarin	122.51	73.30	27.83	27.19	
Lotaustralin	122.14	76.82	24.21	34.00	8.82

Carbon-carbon coupling constants [Hz]:

	(1−2)	(2−3)	(2−4)	(4−5)
Linamarin	60.1	39.2	39.7	
Lotaustralin	59.6	39.9	38.4	34.7

Isotope shifts [Hz]:

	C1	C2a	C2b	C2c	C2e	C3	C4	C5
Linamarin	−0.1	−3.1	−2.8	−0.5	0.0	−1.6	−1.5	
Lotaustralin	0.0	−3.5	−3.3	na[c]	na[c]	−1.5	*	−1.0

* −2.2, −1.7 and −0.7 for species with carbons 2−4−5, 2−4 and 4−5 enriched, respectively

[a] Assignments interchangeable.
[b] In reference [10] the shift for C2 should be 72.92 ppm for linamarin.
[c] na = not available.

australin from *Zygaena* larvae. The intensities of the lines of C-2 of lotaustralin from *Heliconius* larvae are visible but not as easily identifiable due to poorer signal to noise arising from the low yield of lotaustralin in this experiment. This is again direct evidence for incorporation of the intact 85% enriched amino acid with loss of only the carboxyl carbon. Further evidence is afforded by the excellent correspondence of the experimental and theoretical spectra for C-4 of the aglycone moiety of lotaustralin (Fig. 3). Again account was taken of the fractional content of each species and its isotope shift to give the composite spectrum. In this case the degree of enrichment was $1.5 \pm 0.2\%$ for lotaustralin from larvae of *Z. filipendulae* whereas the value of that from larvae of *H. melpomone* was not calculated in view of the low intensity spectrum. The C-13 data for linamarin and lotaustralin from the feeding experiments with *Z. filipendulae* are reported in Table II. Similar results were obtained for *H. melpomone*.

These results clearly show that both, larvae of *Zygaena filipendulae* as well as those of *Heliconius melpomone* incorporate valine into linamarin and isoleucine into lotaustralin specifically without any disruption of the carbon bonds except for loss of the carboxyl group. In this aspect the biosynthesis is similar to that occurring in plants indicating that the pathway used by plants and these Lepidoptera may be very similar, if not the same. Similar results were obtained for some millipeds (Arthropoda: Diplopoda) which synthesize mandelonitrile after feeding with ^{14}C-labelled phenylalanine [11, 12]. Further studies, however, are necessary using different intermediates such as the corresponding nitrile in order to establish the suspected identity in the biosynthesis of cyanogenic glucosides by plants and Lepidoptera.

Acknowledgement

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