*Phytochemistry*, Vol. 31, No. 11, pp. 3839–3843, 1992
Printed in Great Britain.

0031-9422/92 $5.00+0.00


# BIOSYNTHESIS OF IRIDOID GLUCOSIDES IN *HEBENSTRETIA DENTATA*

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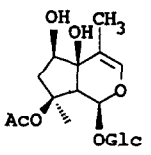
(*Received* 10 *February* 1992)

**Key Word Index**—*Hebenstretia dentata*; Scrophulariaceae; iridoid glucosides; ipolamiide; lamiide; biosynthesis; $^2$H NMR.

**Abstract**—Deuterium-labelled samples of 8-*epi*-iridodial, 8-*epi*-iridotrial and their glucosides were fed to *Hebenstretia dentata*. The aglucones were better precursors than their glucosides of ipolamiide and lamiide, the iridoid glucosides of the plant. A feeding experiment with $^{13}$C-labelled 10-hydroxy-geraniol showed that C-3 and C-11 are equivalent during biosynthesis of the iridoid glucosides and, thus, glucosidation must take place after formation of 8-*epi*-iridotrial. Isolation of the minor metabolites after feeding with 8-*epi*-deoxyloganic acid suggested that the route from this compound proceeds by oxidation at C-8 followed by esterification and that oxidation at C-5 is the final step.

## INTRODUCTION

*Hebenstretia dentata* L.† is an African Scrophulariaceae which contains the iridoid glucosides ipolamiide (**1**) and lamiide (**2**) [2]. We have previously shown that 8-*epi*-deoxyloganin (**10**) is a precursor for **1** and **2** in this plant [2], this being the first report that a compound of the 8-*epi* series can serve as a precursor of an iridoid glucoside. Earlier, the biosynthesis of **1** and **2** was studied in *Lamium amplexicaule* L. (Lamiaceae) which also contains the Me-11 iridoid, lamioside (**14**) [3–5]. The results were, however, not conclusive. A feeding experiment with [2-$^{14}$C]mevalonic acid (**3**) gave low incorporations (0.004–0.024%) into **1**, **2** and **14** [3, 4]. On chemical degradation, $CO_2$ derived from C-11 in **1** and **2** accounted for less than 1% of the specific activity of the compounds. The activity originating from the 3-position, which supposedly should have contained half of the activity, was not measured. Based on these results, it was concluded that the biosynthesis of **1** and **2** proceeds without C-3 and C-11 becoming equivalent and that **6** is a likely intermediate. A subsequent study gave contradictory results, i.e. pointing to a route through **5** and **7** [5]. However, the labelled iridoids used in these experiments contained impurities which could account for the incorporations measured [6]. Thus the previous results do not give a clear picture of the biosynthesis of **1** and **2** in *Lamium*. The aim of the present study was to outline the biosynthetic pathway with some details for **1** and **2** in *Hebenstretia*. Scheme 1 gives some of the possible pathways to **1** and **2** based on the hypothesis that **10** is an intermediate in the biosynthesis. We have prepared and tested a number of deuterium-labelled iridoids likely to be intermediates and have carried out feeding experiments with $^{13}$C-labelled 10-hydroxy-geraniol, to investigate whether C-3 and C-11 become equivalent during the biosynthesis of **1** and **2** in *Hebenstretia*.

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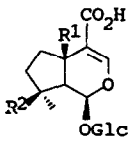
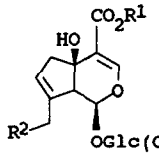



16 $R^1$=OH $R^2$=H

17 $R^1$=$R^2$=OH




18 $R^1$=$R^3$=H, $R^2$=OH

19 $R^1$=Me, $R^2$=OH, $R^3$=H

20 $R^1$=Me, $R^2$=OAc, $R^3$=Ac

21 $R^1$=Me, $R^2$=$R^3$=H

## RESULTS AND DISCUSSION

In the first series of experiments, deuterium labelled analogues of **10** and **5–8** were administered to *H. dentata*, in order to investigate the early steps in the biosynthesis of **1** and **2**. Syntheses of the precursors labelled with deuterium at the 6, 7, 8 and 10 positions have been reported elsewhere [7, 8]. The results of the feeding experiments are shown in Table 1 (Expts 1–5). There was a very good incorporation of **10** into **1** and **2**, with 8-*epi*-iridodial (**5**) and 8-*epi*-iridotrial (**7**) being incorporated into these compounds much better than the respective glucosides **6** and **8**. This indicates that the main biosynthetic pathway to ipolamiide (**1**) and lamiide (**2**) proceeds via the aglucones **5** and **7**. A feeding experiment with 10-hydroxy[9-$^{13}$C]geraniol (c-**15**) confirmed such a hypothesis (Expt. 6), since good incorporations were

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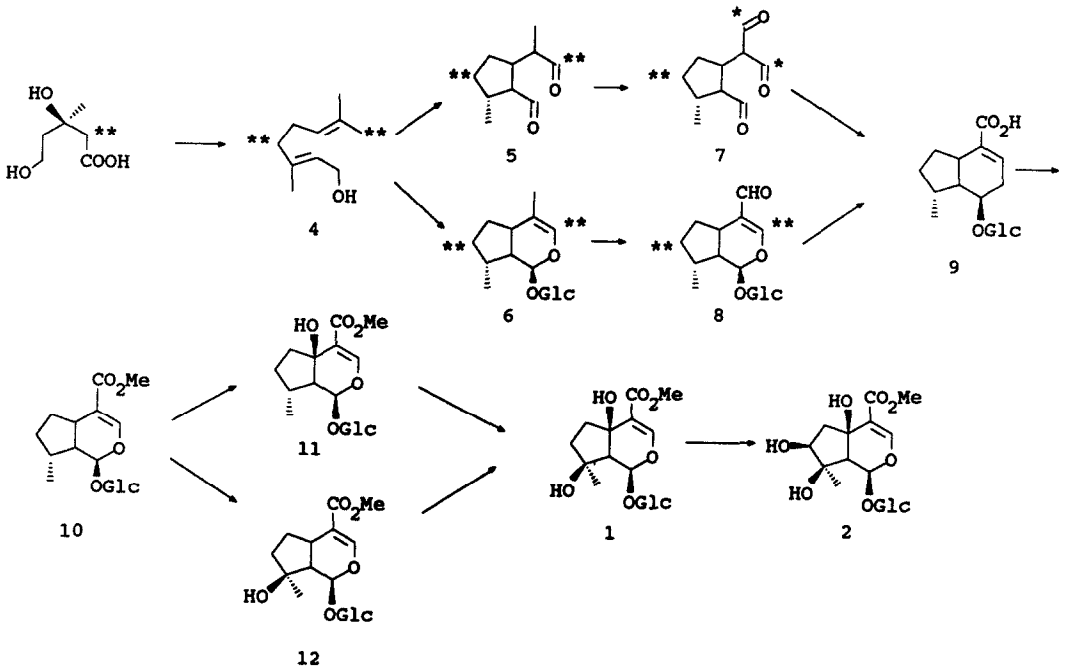
†*Hebenstretia* has been spelt *Hebenstreitia* Murr, but is now acknowledged as *Hebenstretia* L. [1].



Scheme 1. Hypothetical biosynthetic pathways to ipolamiide and lamiide.

Table 1. Administration of labelled precursors to *H. dentata*

| Expt. | Precursor | Incorporation (%) into Ipolamiide (1) | Lamiide (2) |
|---|---|---|---|
| 1 | d-10 | 43 | |
| 2 | d-5 | 4 | |
| 3 | d-6 | 1–2 | |
| 4 | d-7 | 10 | |
| 5 | d-8 | 5 | |
| 6 | c-15 | 1–2* | 1–2* |
| 7 | d-11 | 35 | 3 |
| 8 | d-12 | 50 | 2 |
| 9 | d-1 | (57)† | 5 |

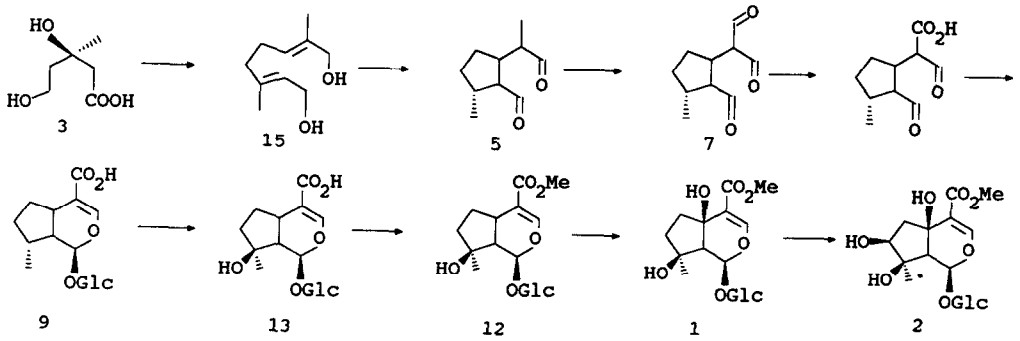
*Scrambling between C-3 and C-11 was observed.
†Re-isolated precursor.

obtained into **1** and **2** and, furthermore, equilibration between C-3 and C-11 was observed in both compounds. This equilibration again suggested the intermediacy of **5** and **7** in the biosynthesis of **1** and **2**.

The notable incorporations seen upon feeding with **6** or **8** can best be explained by hydrolysis of the glucosides after feeding them to the plant, followed by incorporation of the aglucones. Similar results have been reported before [9].

In the next series of experiments, the hydroxylation sequence of the iridoid skeleton was investigated by feeding with [*MeO*-$^2H_3$]-labelled derivatives of mussaenoside (**12**), 5-hydroxy-8-*epi*-deoxyloganin (**11**) and ipolamiide (**1**). Deuterium-labelled **12** was made by treatment of mussaenosidic acid (**13**), isolated from *Utricularia* [10], with $C^2H_2N_2$ in $EtO^2H$. Labelled **1** was prepared by saponification of **1** isolated from *Hebenstretia* and treatment of the acid as above. Unlabelled **11** could be synthesized from theveside (**18**). Methylation ($CH_2N_2$) and acetylation gave **20** which on transfer hydrogenation (Pd/C, $5HCO_2H \cdot 2NEt_3$) yielded **21**. Hydrogenation of **21** over Rh/C gave **11** as the main product. Transformation of **11** to [*MeO*-$^2H_3$]**11** was carried out as above. The results obtained on feeding these compounds to *H. dentata* are shown in Table 1 (Expt 7–9). The incorporation of d-**1** into **2** demonstrates, as expected, that **1** is an intermediate in the biosynthesis of **2**. However, both the 8-hydroxy derivative (**12**) and the 5-hydroxy-derivative (**11**) were found to be good precursors of **1** and it was not possible to deduce the main biosynthetic pathway from these results.

Consequently, we attempted to trap the possible intermediates between 8-*epi*-deoxyloganic acid (**9**) and **1**. There are six possible intermediates between **9** and **1**. Three are acids (**13**, **16** and **17**) and three are methyl esters (**10**–**12**). In the first two experiments, d-**9** was fed to *H. dentata* and the plants were worked-up after 3 and 5 hr, respectively, thus allowing a much shorter time for metabolism of the precursor than in the previous experiments, where the plants were worked-up after 3 days. Aqueous $NaHCO_3$ solution was added to the crude aqueous extracts before application to LPLC. This procedure ensured the fast elution of a fraction containing carbohydrates plus iridoid acids/salts (CIAS-fraction) and thus separation from the iridoid esters which were eluted with normal retention times. Prior to chromatography, small amounts of unlabelled esters **10**–**12** were added to allow detection and isolation of even small amounts of labelled material. In this way **1**, **10**–**12** were



Scheme 2. Probable biosynthetic pathway to ipolamiide and lamiide.

isolated. Lamiide (**2**) was also present but was not isolated in this series of experiments. To facilitate isolation of the remaining metabolites, the CIAS-fraction was neutralized with acetic acid, dissolved in ethanol–water and treated with an excess of diazomethane in ether. Having in this way converted the iridoid acids to methyl esters, a solution containing small amounts of unlabelled **1**, **10**–**12** was added and the mixture chromatographed. Any labelled iridoid acids **9**, **13**, **16** and **17** were thus isolated as their corresponding methyl esters.

In a third experiment, the plants were allowed to metabolize the substrate for 24 hr before work-up and no unlabelled material was added during the isolation procedure.

Table 2 gives the results of the 3 hr experiment (the two other experiments gave similar results). In all cases, a large incorporation of d-**9** into ipolamiide (**1**) was seen, and some of the precursor was re-isolated. Furthermore, d-**9** was incorporated into both mussaenosidic acid (**13**) and mussaenoside (**12**). No incorporation could be detected in 8-*epi*-deoxyloganin (**10**), the 5-hydroxy-compounds **11** and **16**, or in 'ipolamiidic acid' (**17**). This shows that the main biosynthetic route from 8-*epi*-deoxyloganic acid to ipolamiide (**1**) proceeds via **13** and **12**. 8-*epi*-Deoxyloganin (**10**) is, unexpectedly, not on the main path despite the fact that this compound is efficiently converted to **1** in the plant.

Mass spectra of the compounds from the third experiment showed that small amounts of unlabelled mussaenoside (**12**) was present in the plant. Unlabelled 8-*epi*-deoxyloganic acid (**19**) and mussaenosidic acid (**13**) could not be detected. This is most likely due to very small pools of these compounds, although it cannot be excluded that they are not intermediates in the biosynthesis of **1** and **2**. With the above results we are now able to propose a more detailed biosynthetic pathway for the formation of the iridoids in *H. dentata* (Scheme 2). Whether another path to **1** and **2** exists in *Lamium amplexicaule* seems doubtful and a reinvestigation of the biosynthesis in this plant seems warranted.

Table 2. Incorporation of d-**9** into **1** and minor metabolites in *H. dentata*

| | Incorporation (%) | | | |
|---|---|---|---|---|
| Acids | **9** | **13** | **16** | **17** |
| | 2.4* | 3.6 | <0.1 | <0.1 |
| Me-esters | **10** | **12** | **11** | **1** |
| | <0.1 | 1.2 | <0.1 | 14 |

The metabolic period was 3 hr. Experiments with metabolic periods of 5 and 24 hr gave similar results.

* Re-isolated precursor.

## EXPERIMENTAL

Microanalyses were performed by LEO Microanalytical Laboratory, Ballerup, Denmark. Mps: uncorr. Except when otherwise specified, prep. chromatography was performed on Merck Lobar reversed phase columns ($C_{18}$) eluted with the $H_2O$–MeOH mixts specified in each case. Peaks were detected by UV at 238 mm. The $^2H$ NMR spectra were recorded at 76.8 MHz in $H_2O$ or $CHCl_3$ with 0.016 and 0.017% $^2H$ of natural abundance, respectively. Growing specimens of *H. dentata* and *Lantana camara* were obtained from the experimental station of the Botanical Garden of Copenhagen in Tåstrup near Copenhagen.

*Precursor d-***10**. The preparation of d-**10** has been described [7]. The compound contained 0.7 $^2H$ at C-6, 0.9 $^2H$ at C-7, 0.8 $^2H$ at C-8, 0.25 $^2H$ at C-9 and 2.1 $^2H$ at C-10.

*Precursors d-***5**, *d-***6**, *d-***7** *and d-***8**. Prepared as described in ref. [8]. The deuterium content was as above.

10-*Hydroxy* [9-$^{13}$C]*geraniol* (c-**15**). The same product as in ref. [9] was used. An impurity of *ca* 15% of the [10-$^{13}$C]-9-hydroxy epimer was present.

10-*Deoxytheviridoside* (**21**). Theveside (**18**), isolated from *Lantana camara* [11], was dissolved in EtOH and converted to theviridoside (**19**) with ethereal $CH_2N_2$. Acetylation ($Ac_2O$–pyridine, 20°, 2 hr) and purification by prep. TLC (silica gel, $Et_2O$) gave the pure pentaacetate (**20**). To a soln of **20** (498 mg) in EtOH was added 102 mg TEAF (5 $HCO_2H \cdot 2NEt_3$) and 175 mg Pd/C (5%) [8]. The soln was heated to reflux and the reaction monitored by TLC. After 45 min, an additional amount of TEAF (100 mg) was added. The reflux was maintained for a further 45 min period after which the soln was filtered and evapd. The crude reaction mixture was dissolved in 0.1 M NaOMe in MeOH (10 ml). After 1 hr, the deacetylation was complete as judged by HPLC. Addition of HOAc (0.5 ml) and evapn gave a crude product which was applied directly to a B-column. Chromatography with $H_2O$–MeOH (3:1→2:1) gave pure 10-deoxytheviridoside (**21**, 171 mg, 54%) which was characterized by

NMR: $^1$H NMR (500 MHz, $D_2O$): $\delta$7.54 (*s*, H-3), 5.65 (*d*, $J$ = 4.2 Hz, H-1), 5.44 (*br s*, H-7), 3.73 (*s*, OMe), 3.01 (*br s*, H-9), 2.78, 1.71 (each 1H, *br* AB*d*, $J$ = 17 Hz, H-6), 1.73 (3H, *br s*, Me-10); $^{13}$C NMR (63 MHz, $D_2O$): $\delta$96.5 (C-1), 154.7 (C-3), 113.4 (C-4), 75.7 (C-5), 45.9 (C-6), 126.2 (C-7), 137.3 (C-8), 58.1 (C-9), 15.8 (C-10), 169.5 (C-11), 52.7 (OMe), 99.6 (C-1'), 73.4 (C-2'), 76.3 (C-3'), 70.4 (C-4'), 77.3 (C-5'), 61.5 (C-6') (used as int. reference) [12].

5-*Hydroxy*-8-epi-*deoxyloganin* (**11**). 10-Deoxytheviridoside (**21**, 160 mg) was dissolved in EtOH (10 ml) and hydrogenated using Rh/C (10%, 53 mg) as catalyst. After 2 hr the catalyst was removed by filtration and the filtrate concd. Application to a B-column and chromatography with $H_2O$–MeOH (2:1) gave pure **11** (79 mg, 49%) as a foam, $[\alpha]_D^{20}$ −139° (MeOH; *c* 0.9); $^1$H NMR (500 MHz, $D_2O$): $\delta$7.60 (*s*, H-3), 5.80 (*br s*, H-1), 3.80 (*s*, OMe), 2.55 (2H, *m*, H-8 and H-9), 2.29 (*m*, H-6), 2.01 (*m*, H-6), 1.93 (*m*, H-7), 1.22 (*m*, H-7), 0.91 (*d*, $J$ = 6.4 Hz, Me-10); $^{13}$C NMR (63 MHz, $D_2O$): $\delta$96.3 (C-1), 154.1 (C-3), 113.9 (C-4), 73.4 (C-5), 39.4 (C-6), 32.3 (C-7), 34.7 (C-8), 52.2 (C-9), 15.9 (C-10), 169.5 (C-11), 52.6 (OMe), 99.3 (C-1'), 73.2 (C-2'), 76.2 (C-3'), 70.5 (C-4'), 77.2 (C-5'), 61.5 (C-6'). (Found: C, 51.3; H, 7.1 $C_{17}H_{26}O_{10} \cdot \frac{1}{2} H_2O$ requires: C, 51.1; H, 6.8.)

The 8-epimer (24 mg, 15%) was obtained as a by-product and was characterized only by NMR: $^1$H NMR (500 MHz, $D_2O$): $\delta$7.54 (*s*, H-3), 5.67 (*d*, $J$ = 2.2 Hz, H-1), 3.79 (*s*, OMe), 2.16 (2H, *m*, H-6 and H-7), 2.00 (*dd*, $J$ = 9.5 and 2.2 Hz, H-9), 1.76 (2H, *m*, H-6 and H-8), 1.44 (*m*, H-7), 1.14 (3H, *d*, 6.0 Hz, Me-10); $^{13}$C NMR (63 MHz, $D_2O$): $\delta$96.7 (C-1), 153.4 (C-3), 113.7 (C-4), 74.4 (C-5), 39.3 (C-6), 31.4 (C-7), 35.5 (C-8), 57.2 (C-9), 19.9 (C-10), 169.7 (C-11), 52.6 (OMe), 99.7 (C-1'), 73.3 (C-2'), 76.2 (C-3'), 70.5 (C-4'), 77.3 (C-5'), 61.5 (C-6').

5-*Hydroxy*-8-epi-*deoxy*[MeO-$^2H_3$]*loganin* (*d*-**11**). The synthesis of this compound will be reported elsewhere, but it contained a mean of 2.3 $^2$H mol$^{-1}$ as determined by $^1$H NMR. The $^1$H NMR data were as for **11** except for the low intensity of the MeO group.

[MeO-$^2H_3$]*Mussaenoside* (*d*-**12**). Mussaenosidic acid (**13**) was isolated from *Utricularia australis* [10]. The acid (**13**, 340 mg) was evapd twice with $^2H_2O$ (1.5 ml), dissolved in Et$O^2$H (10 ml) and treated with an excess of $C^2H_2N_2$ in $Et_2O$ [13]. After evapn of the solvent, the crude product (350 mg) was applied to a B-column and pure d-**12** isolated after chromatography with $H_2O$–MeOH (5:2). $^1$H NMR data are as for **12** except for the low intensity MeO group. MS showed a label distribution of $^2H_1$, 5%; $^2H_2$, 22% and $^2H_3$, 73%. (calculated from *m/z* 228–231) with a mean of 2.69 $^2$H mol$^{-1}$ (enrichment 90%).

[MeO-$^2H_3$]*Ipolamiide* (*d*-**1**). Ipolamiide (**1**, 58 mg), isolated from *H. dentata* was dissolved in 1M NaOH (5 ml). After 1 day, the soln was neutralized with ion exchange resin (IR 120) and evapd. The acid was evapd twice with $^2H_2O$ (1.5 ml), dissolved in Et$O^2$H and treated with an excess of $C^2H_2N_2$ in $Et_2O$ [13]. After evapn of the solvent, the crude product was applied to a B-column and pure d-**1** (40 mg) isolated after chromatography with $H_2O$–MeOH (3:1). $^1$H NMR data are as for 1 except for the low intensity of the MeO group. MS showed an approximate distribution of label of $^2H_1$, 10%; $^2H_2$, 27% and $^2H_3$, 63% (calculated from the absorptions *m/z* 245–247) with a mean of 2.5 $^2$H mol$^{-1}$ (enrichment 85%).

8-epi-*Deoxy*[6,7,8,10-$^2$H]*loganic acid* (*d*-**9**). The compound described in ref. [7] was used. It contained 0.8 $^2$H at C-6, 0.6 $^2$H at C-7, 0.7 $^2$H at C-8 and 2.0 $^2$H at C-10.

*General procedure for the administration of labelled precursors and for the isolation of iridoids.* The precursor was dissolved in $H_2O$ (3–5 ml). In experiments with aglucones, 10 $\mu$l of a detergent [9] was added to improve uptake. The freshly cut stems were immersed in the soln/emulsion of precursor. Absorption was usually complete within 3 hr, after which time more $H_2O$ was added. Except when otherwise specified, the plant material was harvested after 3 days, blended with EtOH, the solvent evapd and the residue partitioned between $H_2O$ and $Et_2O$. Evapn of the aq. fr. gave a crude extract which on application to a B-column and elution with $H_2O$–MeOH (4:1→3:1) gave lamiide (**2**) and ipolamiide (**1**). The amounts of precursor, plant material, etc. are given in Table 3.

*Short-time experiments with d-***9**. In the first two experiments, the plant material was worked-up after 3 and 5 hr, respectively. The crude extract was treated with act. C (100 mg) and dissolved in satd aq. $NaHCO_3$ (1 ml) and 1 ml of an aq. soln of authentic **10**, **11** and **12** (*ca* 5 mg of each) was added. The resulting soln was applied to a B-column and eluted with $H_2O$–MeOH (8 ml min$^{-1}$) 5:1 for 30 min, 3:1 for 40 min and 1:1 for 30 min. Evapn of the 5:1 eluate gave a fr. containing carbohydrates and

Table 3. Feeding experiments

| Precursor | | Plant material | Crude extract | Ipolamiide (**1**) | Lamiide (**2**) |
|---|---|---|---|---|---|
| Compound | Amount (mg) | (g) | (mg) | (mg) | (mg) |
| d-**10**[e] | 24.1 | 26 | 630 | 82 | 12 |
| d-**5**[e] | 9.2 | 31 | 610 | 60 | 11 |
| d-**6**[e] | 21.0 | 33 | ~600 | 51 | 7 |
| d-**7**[e] | 11.3 | 29 | 670 | 58 | 10 |
| d-**8**[e] | 19.3 | 33 | 860 | 97 | 33 |
| c-**15**[e] | 13.0 | 6.2 | 150 | 15 | 7 |
| d-**11** | 25.1 | 22 | 550 | 83 | 14 |
| d-**12** | 24.0 | 24 | 508 | 86 | 7 |
| d-**1** | 21.2 | 24 | 503 | 71 | 6 |
| d-**9**$^{3}$* | 17.3 | 10 | 246 | 36 | ni† |
| d-**9**$^{5}$ | 17.3 | 10 | 220 | 45 | ni |
| d-**9**$^{24}$ | 17.3 | 9 | 221 | 34 | ni |

[e] Emulsifier added.

* The superscript indicates the metabolic period in hr.

†ni: Not isolated.

iridoid acids (CIAS fr.). Lamiide (**2**), ipolamiid (**1**) and mussaenoside (**12**) were eluted with 3:1 and **11** and **10** with 1:1. This procedure allowed baseline separation of these iridoids.

The CIAS-fr. was neutralized with HOAc, dissolved in EtOH–$H_2O$ and treated with an excess of $CH_2N_2$ in $Et_2O$. Excess $CH_2N_2$ was destroyed with HOAc and the soln evapd. After repetition of the methylation, a soln containing authentic **1**, **10**, **11** and **12** (*ca* 5 mg of each) was added. Chromatography on a B-column with $H_2O$–MeOH (3:1→1:1) as above gave the pure iridoids.

In the third experiment, the plant material was worked-up after 24 hr. Unlabelled iridoids were not added during the work-up procedure. Small amounts (< 1 mg) of **9**, **13** and **12** were isolated (**9** and **13** isolated as their methyl esters **10** and **12**). $^1$H NMR showed that the compounds were mainly or exclusively deuterium labelled. MS of the glucosides showed *ca* 10% unlabelled material in **12**, whereas no significant amounts of unlabelled material could be detected in the two other samples.

*Calculation of incorporations from NMR experiments with deuterium-labelled precursors.* In the experiments with methoxy-$^2H_3$-labelled precursors, the incorporations were calculated from the proportion between the integral of the $^1H^2HO$ peak of natural abundance and the peak at $\delta$3.7 in the $^2$H NMR spectrum.

In the experiments with multiple deuterium labelled precursors, the incorporations were calculated from the proportion between the integral of the $^1H^2HO$ peak and the 10-Me peak. In all cases, chemical shifts of the retained C-6 and C-7 deuteriums in **1** and **12** were within 0.1 ppm of the chemical shifts seen in the $^1$H NMR spectra of the same compounds.

*Feeding experiment with* 10-*hydroxy* [9-$^{13}$C]*geraniol* (*c*-**15**). This experiment was performed in October, in contrast to the others which were carried out during spring or summer. Early in the year, *H. dentata* contained **1** and **2** in typically 5:1 to 10:1 proportion, whereas the proportion in the autumn was *ca* 2:1. The incorporations and enrichments in the experiment with c-**15** were estimated by comparison of the $^{13}$C NMR peak heights of C-3 and C-11 with the mean peak heights of C-4, C-1′, C-1, C-5′, C-3′–C-2′ and C-4′. In the spectrum of **1**, the enrichment was 3.0% at C-3 and 2.5% at C-11. In the spectrum of **2** the enrichment was 3.9% at C-3 and 4.6% at C-11. The two pairs of C-3/C-11 were identical within the error of measurement. The higher enrichment in **2** may be due to the increased rate of biosynthesis of **2** late in the year. In view of the large enrichments, the presence of the impurity of the 9-hydroxy[10-$^{13}$C]-isomer was judged to be unimportant.

*Acknowledgements*—We thank the Danish Natural Science Research Council and the Carlsberg Foundation for access to NMR facilities, LEO, Denmark for microanalysis, and the staff of the Botanical Garden of the University of Copenhagen for growing the plant material. We also thank Brdr. Hartmanns Foundation for the $^2$H NMR probe.

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