Phytochemistry, Vol. 28, No. 1, pp. 97–105, 1989.
Printed in Great Britain.

0031-9422/89 $3.00+0.00

BIOSYNTHESIS OF THE IRIDOID GLUCOSIDE CORNIN IN *VERBENA OFFICINALIS*

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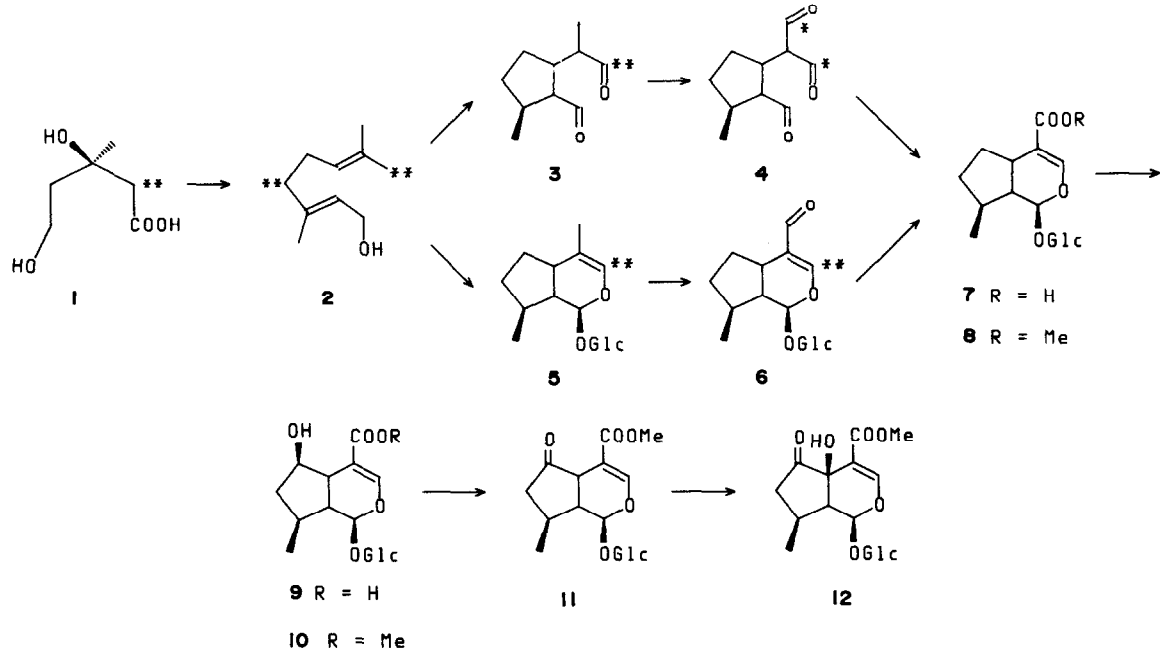
(*Received* 6 *May* 1988)

Key Word Index—*Verbena officinalis*; Verbenaceae; cornin; dihydrocornin; iridoid glucosides; biosynthesis; ^{13}C-labelling; ^{2}H-labelling.

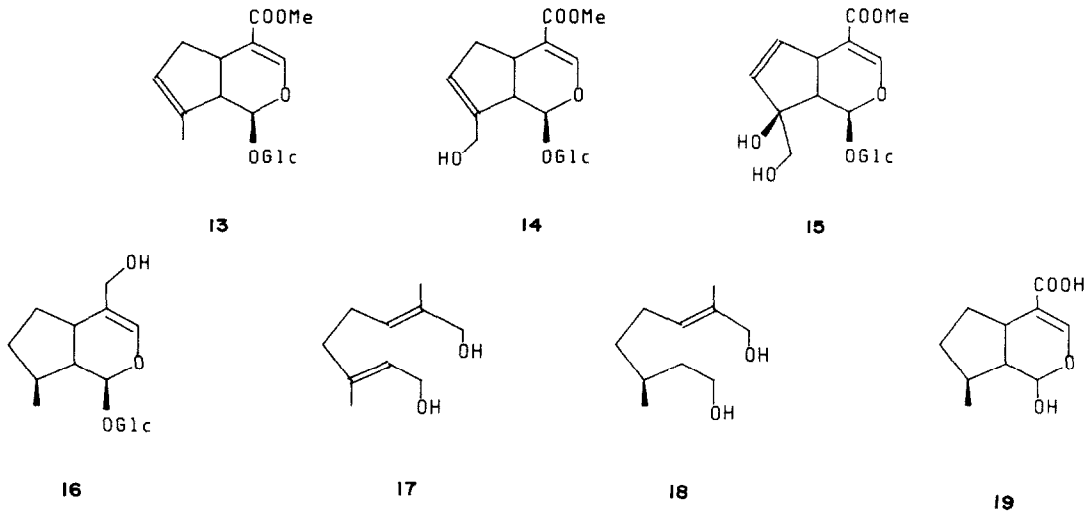
Abstract—The biosynthesis of cornin (verbenalin) and dihydrocornin *in Verbena officinalis* has been investigated. The incorporation of [^{2}H] deoxyloganin was found to be largely independent of the incubation time between one day and a week. An improved method for the preparation of deoxygeniposide from gardenoside is reported and [^{2}H]-iridodial glucoside and [^{2}H]-iridotrial glucoside were prepared from the former. Feeding experiments with young plants using these glucosides, as well as the aglucones, showed much better incorporations for the latter compounds as measured by ^{2}H NMR spectroscopy. ^{13}C-labelled 10-hydroxygeraniol, 10-hydroxycitronellol, iridodial, iridotrial, iridodial glucoside, iridotrial glucoside, deoxyloganic acid, and deoxyloganic acid aglucone were prepared. [^{13}C]-Mevalonic acid and the above compounds were fed to plants of medium age, and all gave incorporations measurable by ^{13}C NMR spectroscopy into dihydrocornin. The postulated existence of two different metabolic pathways in the biosynthesis of cornin in young and old plants, respectively, could not be established as complete scrambling between the C-3 and C-11 in the iridoid skeleton apparently takes place with all the early precursors. The complete pathway from iridodial forwards to hastatoside has been elucidated.

INTRODUCTION

The biosynthesis of iridoid compounds has been much investigated [1]. Early experiments [2, 3] dealing with the formation of the iridoid glucoside cornin (verbenalin) in *Verbena officinalis* proved the mevalonoid origin of this compound, although only a poor incorporation of geraniol was obtained which was probably due to poor translocation of the precursor in the plant [3]. In feeding experiments with [2-^{14}C]-mevalonate it was furthermore shown that a variable degree of scrambling of the labelling took place, depending on the age of the plants used, the scrambling phenomenon is demonstrated in Scheme 1. Dependent on whether glucosylation takes



Scheme 1. Possible biosynthetic pathways to cornin.



13 14 15 16 17 18 19

place before or after C-3 and C-11 reach the same oxidation state and thus become equivalent, the labelling from C-2 in mevalonic acid (**1**) will be found solely at C-3 or distributed evenly between C-3 and C-11. This age-dependent degree of scrambling was explained by the existence of two different biosynthetic pathways in young and mature plants. Such a result is very interesting, since scrambling apparently takes place in the biosynthesis of most other iridoids investigated [cf. 1]. The later stages of the formation of cornin (**11**) have been investigated extensively. Thus, deoxyloganic acid (**7**) gave an incorporation of 11% into **11** [4], while deoxyloganin (**8**) gave 5% [5], both in *V. officinalis*. Likewise, **8** and dihydrocornin (**10**) were both incorporated into **11** (3 and 2%, respectively) and hastatoside (**12**, 1 and 0.5%, respectively) in *V. hastata* [5]. Furthermore, it has been shown [6, 7] that when feeding **8** to *V. officinalis* much of the labelling resides in dihydrocornin (**10**), and that this compound is an intermediate in the formation of **11**. Contrary to this, reisolated **8** was not diluted by unlabelled material, and therefore **8** was considered an unlikely intermediate in the formation of **10** and **11**. Similarly, a single experiment [6] has shown incorporation (0.7%) of iridodial glucoside (**5**) into **11**, but again, recovered **5** was not diluted and thus an unlikely intermediate. In order to further clarify the early steps of the biosynthesis of the iridoids in *V. officinalis*, we have prepared and tested a number of labelled compounds likely to be intermediates.

RESULTS AND DISCUSSION

Syntheses of labelled compounds

Preparation of deoxyloganic acid (**7**) labelled with ^3H at C-10 and of deoxyloganin (**8**) labelled with ^2H at the methoxy group have been described [4, 5]. In both cases the compounds were prepared from geniposide pentaacetate (**14a**)* by catalytic hydrogenation. As we have had difficulty in obtaining sufficient amounts of **14a**, we have developed an alternative synthesis of deoxygeniposide (**13**) from gardenoside hexaacetate (**15a**) by catalytic transfer hydrogenation using formic acid/palladium on carbon [8, 9] in 1,4-dioxane. The crude reaction product from the above reaction was deacetylated and pure deoxygeniposide (**13**) obtained in a 67% yield by reversed phase chromatography. Catalytic hydrogenation of **13** with palladium on carbon as the catalyst then produced deoxyloganin as the main product although a considerable amount of the 8-epimer (*ca* 20%) was present.

For the present work we decided again to test the optimum incubation time for the experiments. Despite the fact that deoxyloganin (**8**) is probably not an intermediate, it is easy to prepare with a ^2H label in the carbomethoxy group, and in addition the incorporation is simple to measure [5–7]. Thus deoxyloganic acid was treated with [$^2H_2$]-diazomethane to give labelled deoxyloganin (d_3-**8**), and this was purified by preparative HPLC to give an isomer-free (< 1% by HPLC) preparation.

The preparation of iridodial and iridotrial glucoside (**5** and **6**) from **8** by reduction of **8a** with lithium aluminium hydride followed by either catalytic hydrogenation or oxidation has been described in detail [10], and has been followed here. Thus the initial catalytic transfer hydrogenation of **15a** was performed with [$^2H_2$]-formic acid to give [6,10-$^2H_2$]-deoxygeniposide (d_2-**13**), and catalytic hydrogenation with $^2H_2$ provided [6,7,8,10-$^2H_4$]-deoxyloganin (d_4-**8**), containing 20% of the 8-epimer (but see below). Acetylation followed by reduction with lithium aluminium hydride gave 11-hydroxy-[6,7,8,10-$^2H_4$]-iridodialglucoside (d_4-**16**) in 48% yield. Oxidation with oxygen catalysed by platinum gave [6,7,8,10-$^2H_4$]-iridotrial glucoside (d_4-**6**) in almost quantitative yield. Acetylation of d_4-**16** gave the pentaacetate and catalytic transfer hydrogenation using triethyl amine-formic acid-/palladium on carbon [6] provided [6,7,8,10-$^2H_4$]-iridodial glucoside (d_4-**5**) in 69% yield, catalytic hydrogenation being too unreliable in our hands with over-reduction occasionally taking place. Analysis of the ^2H-content by NMR spectroscopy (see Experimental) showed that deuterium was indeed incorporated in the expected positions, but that considerable scrambling had taken place with *ca* 0.7 ^2H at C-6, 1.9 ^2H at C-7 and C-8

* Fully acetylated glucosides are given the same number as the corresponding glucoside using the suffix *a*. Labelled compounds are numbered likewise, but with a prefix: *d*- for ^2H and *c*- for ^{13}C.

and finally 2.0 ^2H at C-10. A similar labelling would be expected in d_4-**6**. Finally, HPLC-analysis showed that 20% of the 8-epimer was present in each preparation.

In a separate paper [9] we have described the preparation of [3,11-^{13}C]-iridotrial glucoside (*c*-**6**) by a Vilsmeier formylation with *N*,*N*-dimethyl-[^{13}C]-formamide of (8*S*)-6,10-dideoxy-7,8-dihydroaucubin tetraacetate, the latter being obtained stereochemically pure and in reasonable yield from aucubin hexaacetate. Some unexplained scrambling takes place during the synthesis and the product thus contains *ca* 5% ^{13}C at position C-3 and 85% ^{13}C at position C-11. Reduction of *c*-**6** with sodium borohydride followed by hydrogenation as above provided similarly labelled [3,11-^{13}C]-iridodial glucoside (*c*-**5**). Oxidation of *c*-**6a** was performed with sodium chlorite in *tert*-butyl alcohol [11] to give [3,11-^{13}C]-deoxyloganic acid (*c*-**7**) in almost quantitative yield after deacetylation. The aglucones of the above labelled compounds were prepared by incubating with β-glucosidase (see Experimental).

In order to test the incorporation of labelled acyclic terpenoids we have prepared 10-hydroxy-geraniol (**17**), a compound known to be incorporated in other iridoids [1]. Recently, 10-hydroxycitronellol (**18**) was found to be a good precursor of dolicholactone in *Teucrium marum* and nepetalactone in *Nepeta cataria* (both Lamiaceae) [12, 13]. Since Verbenaceae is closely related to Lamiaceae, it seemed appropriate also to test **18** as a precursor for cornin in *Verbena*. The preparation of 10-hydroxy-[9-^{13}C]-geraniol (*c*-**17**) and (3*S*)-10-hydroxy-[9-^{13}C]-citronellol (*c*-**18**) has been described in detail [14]. The syntheses of the two compounds was thus performed essentially as reported, except that we have improved the utilisation of the [^{13}C]-methyl iodide used to introduce the labelling (see Experimental). The products obtained, however, apparently did not show the same isomeric purity as that reported earlier [14], since they both contained *ca* 15% of the 9-hydroxy-[10-^{13}C]-isomers as measured by ^{13}C NMR spectroscopy. In view of the results obtained below, the presence of the additional isomers was judged to be unimportant.

Biosynthetic experiments

As mentioned above, we decided again to test the optimum metabolic period for the experiments using cut plants just before flowering (late June 1985, 1.5–2 months of age). Deoxyloganin (d_3-**8**, *ca* 10 mg in 5 ml of water) was fed to each batch of plants (*ca* 10 g). After eight to twelve hours all of the precursor had been absorbed and the plants were placed in a larger container and left for the prescribed time (1, 2, 3 or 7 days, see Table 1). After work-up (see Experimental) the extract was chromatographed and cornin and dihydrocornin were isolated. After acetylation of the glucosides, the ^2H NMR spectra (38.4 MHz, $CHCl_3$) were recorded and the incorporation calculated, based on the proportion between the OC^2H_3-peak (3.7 ppm) and the natural abundance peak of the C^2HCl_3, essentially as reported earlier [5]. The values found are listed in Table 1.

The present results show that when using plants just before flowering the metabolic period is not very important. The shortest possible metabolic period might seem an advantage, but since we planned to use precursors biosynthetically less advanced than deoxyloganin, and since translocation of compounds with poorer water solubility might take a longer time, we decided to use a metabolic period of three days in the following experiments.

When feeding water-insoluble compounds, these are usually emulsified in water with the addition of Tween 80. However, it appeared that the plants did not tolerate this emulsifier as they died within one day when it was added to the water in a control experiment. After testing a few other emulsifiers, we found that dish-washing detergent was satisfactory for the present purpose (see Experimental).

The second series of experiments was performed with ^2H-labelled iridodial (d_4-**3**), iridotrial (d_4-**4**) and the corresponding glucosides (d_4-**5** and d_4-**6**). The time was in early August, and the plants used were regrowth from flowering plants that had been cut down in July. In this case the incorporations were calculated from the intensities of the 10-CH^2H_2-peak in the NMR spectra. The results are listed in Table 2. Remarkably, iridodial and particularly iridotrial were much better incorporated than the corresponding glucosides, although the incorporations of the latter were not negligible. This could either be interpreted as showing the existence of the two different postulated [3] pathways, or merely that part of the glucoside fed had been hydrolysed by the plant and incorporated as the aglucone. The latter explanation is consistent with the earlier reported experiment [6] where reisolated **5** was found to be undiluted. Furthermore, 4 mg of labelled 11-hydroxy-iridodial glucoside (d_4-**16**) was isolated from the plant fed with d_4-**6** (Experiment 8), corresponding to a conversion of 33%. Since a reduction of **6** had taken place instead of the expected oxidation, this finding indicates that iridotrial glucoside is foreign to the plant and thus not on the biosynthetic pathway. However, feeding experiments designed to determine if complete or partial scrambling between C-3 and C-11 takes place during biosynthesis seemed to be the only safe way to ascertain whether the **5** and/or **6** were intermediates or not.

Table 1. Administration of [OMe-$^2H_3$]-deoxyloganin (d_3-**8**) to *Verbena officinalis* showing the time dependence of incorporation into compounds **10** and **11**

Experiment	Time (days)	d_3-**8** (mg)	Plant (g)	Amount isolated (mg) **10**	**11**	Incorporation (%) into **10**	**11**
1	1	10.5	10.4	6	120	24	33
2	2	10.3	10.1	3	111	20	28
3	3	10.6	10.1	10	115	19	34
4	7	10.9	10.3	8	108	14	27

Table 2. Administration of deuterium labelled precursors to *Verbena officinalis*

Experiment	Precursor fed (mg)	Plant (g)	Amount isolated (mg) 10	11	% Incorporation (% enrichment) 10	11
5	d_4-**3** (8)	19.0	16	202	9 (11)	13 (1.2)
6	d_4-**4** (13)	22.0	26	233	30 (32)	12 (1.4)
7	d_4-**5** (14)	9.2	18	168	4 (4)	5 (0.5)
8	d_4-**6** (12)	9.3	16	167*	3 (3)	3 (0.3)

*d_4-**16** (4 mg) was also isolated from this experiment.

Thus a third series of feeding experiments was planned with the same precursors labelled with ^{13}C in the C-11 position. The plants used in the experiments were of intermediate age when some of them had started to flower (15–25 cm). In order to test for other possible precursors earlier and later on the biosynthetic pathway than **3**–**6**, the labelled compounds prepared above (**1**, **17**–**19** and **7**) were included in this series.

In order to measure accurately the incorporation in a compound by ^{13}CNMR spectroscopy, the content of labelling from the converted precursor must be similar to that of natural abundance to give a doubling of the peak size for the carbon(s) in question. In other words, the enrichment of ^{13}C in the compound from a biosynthetic experiment must be at least similar to the natural abundance (1.1%). The results from the initial series with early precursors (Table 2) showed only minor enrichments for cornin (**11**), but they were considerable for dihydrocornin (**10**) due to the much smaller natural pool of the latter compound. Thus meaningful results could be expected under similar conditions.

These experiments were performed again late in June but the plants did not keep very well when taken indoors to the laboratory, and thus the incorporations possibly were not optimal (when compared with those found in the previous year). All the precursors tested were labelled with ^{13}C and the incorporations were determined by NMR spectroscopy.

The ^{13}CNMR spectra were recorded at 125 MHz and compared with reference spectra of unlabelled **10** or **11**. In each spectrum the mean value of the intensity of the peaks arising from the glucosyl moiety was used as the standard, as labelling was unlikely to be incorporated into these positions. Dividing all peak intensities by this mean value provided the normalized spectra. Comparison with the normalized reference spectrum showed in which positions incorporation had taken place, and for these the enrichment and the incorporation were calculated. The results have been listed in Table 3. The uncertainty is dependent on the signal to noise ratio in each spectrum. Based on the peaks without incorporation, we have found this to be fairly constant in the interval 0.10–0.20 for the enrichments listed in Table 3. This is also the lower limit for determination of the enrichment. However, the uncertainty in the measurement of the intensity of the C-11 signal is probably significantly larger due to the much longer relaxation time for this carbon. Since the incorporated label was evenly distributed between C-3 and C-11 in the isolated **10** and **11** in the cases where scrambling had taken place, the incorporations were calculated as if the same had been the case for the precursors. Using this method, complete purification of the isolated compounds was unnecessary, since the exact amount of **10** was measured by HPLC (see Experimental) and since the impurities did not interfere seriously with the interpretation of the ^{13}CNMR spectra.

The results in Table 3 show that sufficient enrichment was obtained in all cases for the incorporation to be measurable in dihydrocornin (**10**). The large pool of cornin (**11**) present in the plants, however, caused so much dilution that the enrichment in this compound was too small to be determined in most cases. Interestingly, even [2-^{13}C]-mevalonic acid (*c*-**1**, experiment 9), although very early on the biosynthetic pathway, was incorporated to a measurable degree in **10**. An incorporation of *ca* 0.2% was found with the labelling incorporated in the expected positions, namely a little more than half at C-7 and a little less than a fourth each at C-3 and C-11 as seen by the increase of the appropriate peaks at δ41.8, 152.9 and 170.6. The incorporation was not very large, but due to the large amount of precursor fed, the enrichment was considerable. However, most important was the fact that this experiment unambiguously showed that complete scrambling of C-3 and C-11 had taken place. In the first paper on the biosynthesis of cornin, Hüni *et al.* [2] found similarly large incorporations of [2-^{14}C]-mevalonic acid when using much shorter incubation times (2–5 hr) and very young plants (5–7 cm). We tried in an experiment similar to experiment 9 to harvest the plants after only five hours, but no measurable enrichment could be seen in neither **10** nor **11**. This was probably due to the large amount of precursor fed compared with the experiments of Hüni *et al.* [2] who were using a radioactive tracer on a much smaller scale.

In the next two experiments (10 and 11) with 10-hydroxy-[9-^{13}C]-geraniol and -citronellol (*c*-**17** and *c*-**18**, respectively) considerable incorporation in both **10** (4%) and **11** (3%) was seen for the former but not for the latter, where only a trace of the labelling was recovered in **10**. As with mevalonic acid (**1**), compound **17** is incorporated with complete scrambling. As mentioned above, 10-hydroxy-citronellol has been found to be a good precursor for dolicholactone and nepetalactone [12, 13], non-glucosidic iridoids found in plants from the closely related family Lamiaceae, but apparently with a biosynthetic pathway different from that of the glucosides.

The feedings with iridodial, iridotrial and their glucosides (*c*-**3**–*c*-**6**) all gave incorporations into dihydrocornin although in much smaller scale (0.6–2%) than that found the year before (Table 2), probably due to the above-mentioned problem with taking the plants indoors this summer (also the plants in the present series were older).

Table 3. Administration of ^{13}C-labelled precursors to *Verbena officinalis*

Experiment	Precursor fed (mg)	Plant (g)	Compound no.	Isolated mg	% Incorporation (% enrichment) at C-3	at C-11
9	*c*-1 (35.1)	10.5	**10**	1.5	0.15 (2.5)	0.15 (2.4)†
			11	76	—	—
10	*c*-17 (14.1)	10.0	**10**	4.3	4.6 (14)	3.3 (10)
			11	106	1.8 (0.2)	4.0 (0.5)
11	*c*-18 (14.5)*	10.0	**10**	2.1	0.16 (1.1)	0.12 (0.8)
			11	88	—	—
12	*c*-3 (9.5)	9.0	**10**	2.3	1.1 (4.9)	0.9 (4.0)
			11	76	—	—
13	*c*-4 (8.0)	10.0	**10**	1.5	1.5 (8.1)	1.4 (7.3)
			11	80	1.3 (0.12)	1.7 (0.17)
14	*c*-5 (20.0)*	17.5	**10**	4.0	2.0 (5.4)	1.7 (4.6)
			11	187	—	
15	*c*-6 (12.5)*	10.5	**10**	1.7	0.52 (2.0)	0.68 (2.6)
			11	97	—	—
16	*c*-19 (12.5)	10.1	**10**	1.7	2.1 (1.7)	1.7 (21)
			11	63	—	1.5 (0.5)
17	*c*-7 (12.9)	10.5	**10**	2.6	8.2 (2.1)	8.4 (37)
			11	94	12 (0.1)	12 (1.6)

*Unchanged (experiment 14 and 15) or glucosylated precursor (experiment 11) was isolated in these experiments (see Experimental). The incorporations are not corrected for this reason.

†In this case a 0.20% incorporation was found at C-7 (the enrichment was 6.5%).

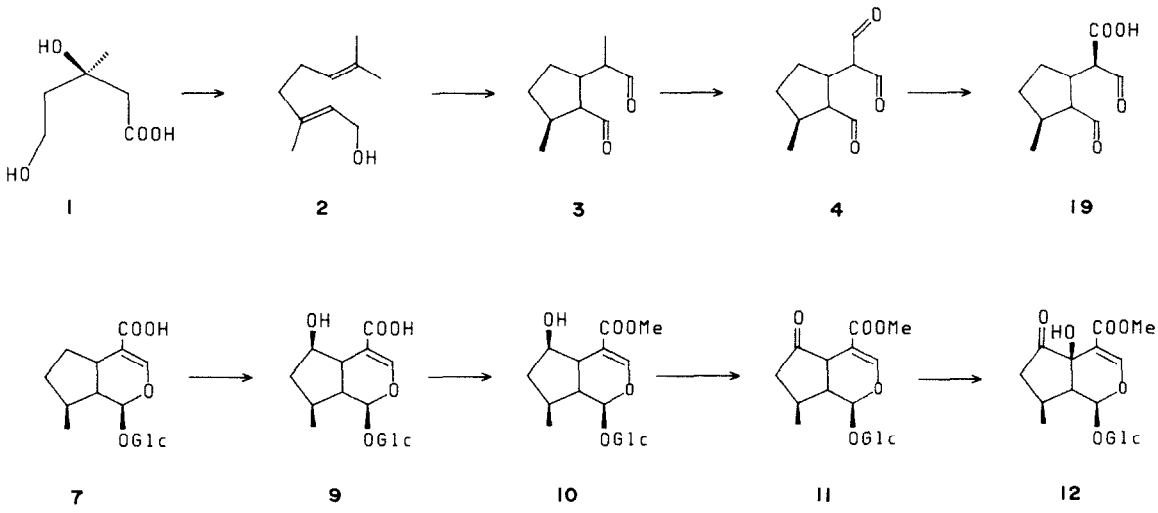
The finding that complete scrambling between C-3 and C-11 had taken place was in accord with the earlier results. Furthermore, 24% of undiluted iridodial glucosides was recovered (experiment 14) and likewise 14 and 58% of the iridotrial glucoside fed (experiment 15) was recovered unchanged or reduced, respectively, indicating that these glucosides were foreign to the plants.

In the last two experiments were used deoxyloganic acid (*c*-**7**) and the corresponding aglucone (*c*-**19**) both prepared from *c*-**6** and thus with identical enrichment. Similar to the earlier reported experiment [4] considerable incorporations with 8 and 12% into **10** and **11**, respectively, were found for *c*-**7**. As expected no scrambling had occurred as the proportions in the enrichments measured for C-3 and C-11 were identical in precursor and products. The incorporations found for the aglucone were unexpectedly small if this compound should indeed be an intermediate on the biosynthetic pathway, namely 2 and 1.5% into **10** and **11**, respectively. Again no scrambling had taken place.

The presence of up to 20% of isomeric impurities in d_4-**3**-d_4-**6** as well as in *c*-17 and *c*-**18** is not considered to be significant in the present work. Firstly, isomer-free preparations of **3**–**6** were used in the last series of experiments and here provided satisfactory incorporations. Secondly, the complete scrambling found in all of the relevant experiments removes any ambiguities that could have been caused by the isomers present in *c*-**17** and *c*-**18**.

In addition to the above experiments, we decided to examine *V. officinalis* more thoroughly concerning the presence of minor constituents. We therefore extracted a large amount (1 kg) of plant material, isolated the major part of the cornin present, and investigated the remaining aqueous extract for minor glucosidic constituents (see Experimental). In addition to **11** (0.5%), we isolated minor amounts of **7** (0.0005%), **10** (0.01%) and **12** (0.005%). The possible presence of **9** could not be determined as the retention time was too close to that of **11**. However, no deoxyloganin could be detected (neither by HPLC nor with ^1H NMR) in the small fraction with the appropriate retention time.

With the above results in hand we are now able to propose a rather detailed biosynthetic pathway concerning the formation of the iridoids in *V. officinalis* (Scheme 2). The known iridoid pathway through mevalonic acid (**1**) and geraniol (**2**) followed by an oxidation of the latter to 10-oxogeranial seems highly probable in view of the good incorporations found for **1** and **17**. Cyclization of 10-oxogeranial to iridodial (**3**) followed by oxidation of the latter to iridotrial (**4**) are then the next steps as neither of the alternative glucosides **5** and **6** are present in the plant, since labelled compounds fed can be reisolated without dilution. Also the scrambling found in feeding experiments using precursors earlier on the pathway than iridotrial exclude these glucosides as precursors. Despite the low incorporation of deoxyloganic acid aglucone (**19**) found in the present work, this compound is highly probable as an intermediate, since it is the only logical step between iridotrial and deoxyloganic acid (**7**), a true intermediate isolated from the plant. The next step is most probably an oxidation of **7** to dihydrocorninic acid (**9**), since the alternative, a methylation to deoxyloganin (**8**) can be excluded. The latter compound has not been detected in the plant, but more importantly, it has been isolated in undiluted form after feeding experiments [6], and must therefore be excluded as an intermediate. The last steps on the pathway are then methylation of **9** to dihydrocornin (**10**) followed by oxidation to cornin (**11**) and finally an additional oxidation of a small portion to yield hastatoside (**12**).



Scheme 2. Probable biosynthetic pathway to dihydrocornin, cornin and hastatoside.

The existence of alternative pathways proposed earlier [3] was necessary in order to explain the results where more or less age-dependent scrambling had been found. A similar phenomenon was seen by the same group [15] when working on the biosynthesis of nepetalactone in *Nepeta cataria*. In recent work [13], however, no such scrambling was found, leaving the earlier results largely unexplained.

EXPERIMENTAL

Microanalyses were performed by NOVO Microanalytical Laboratories, Bagsværd, Denmark. Mps: corr. ^2H_2O (Stohler) and $^2HCO_2{}^2H$ (Ciba-Geigy, *ca* 95% in ^2H_2O) were 99.8 and 99.5%, respectively, in ^2H. All analytical (HPLC) and preparative chromatography was performed on reversed phase columns. For analytical HPLC a 100 × 4.8 mm column packed with Nucleosil 5 C-18 was used. Merck Lobar C-8 columns size A, B or C as well as a larger (2 kg, size D) home-made C-18 column were used for prep. chromatography. Difficult separations on a preparative scale were performed on Merck Hibar 250–25 (LiChrosorb RP-18 (7 my). H_2O–MeOH mixtures were used for elution and the proportion is given in each case. Peaks were detected by UV simultaneously at 254 and 206 nm unless otherwise noted. The ^2H NMR spectra were recorded at 38.4 MHz in $CHCl_3$ with 0.016% ^2H of natural abundance [5].

Growing specimens of *V. officinalis* were obtained from the experimental station of The Botanical Garden of Copenhagen in Tåstrup near Copenhagen. *Verbena officinalis* is a perennial and shoots for the experiments were cut either from seed plants or from the fresh growth of older plants (no difference between the biosynthetic capacity of these shoots could be detected). Foliage of *Gardenia jasminoides* for the isolation of gardenoside was obtained from a commercial gardener. *Chaenostoma foetidum* Bent. (Scrophulariaceae) for the isolation of iridotrial glucoside (**6**) was grown from seeds obtained from The Botanical Garden of Copenhagen.

Deoxygeniposide (**13**). Gardenoside hexaacetate (**15a**, 2.0 g) was dissolved in 1,4-dioxane (20 ml) and filtered through active C (2 g). Pd-catalyst (5% on C, 0.5 g) was added and the mixt. heated to reflux. Formic acid (300 mg) was added with stirring in 5 portions during 10 min. The progress of the reaction was monitored by TLC (when sprayed with dil H_2SO_4 followed by heating **15a** gives a distinct blue colour whereas the products turn red); after *ca* 1 hr all **15a** had disappeared and the mixture was filtered. The solvent was removed and the residue evapd twice with toluene (50 ml) in order to remove AcOH and HCO_2H. The crude product (**13a**) was deacetylated in dry MeOH with MeONa (*ca* 30 min at room temp.) until TLC showed the reaction to be complete. Preparative chromatography (Lobar size C; H_2O–MeOH 2:1; 22 ml/min) gave pure deoxygeniposide (**13**, 760 mg, 67%). Crystallization from 99% EtOH gave a product mp 167–168°. ^1H NMR spectrum (500 MHz, D_2O): δ 7.53 (*br s*, H-3), 5.57 (*m*, H-7), 5.49 (*d*, J = 4.7 Hz, H-1), 4.83 (*d*, J = 8.3 Hz, H-1'), 3.76 (*s*, OMe), 3.21 (*dt*, J = 4.6 and 8 Hz, H-5), 2.90 (*m*, H-9), 2.75 (*br ddd*, J = 1.5, 7.5 and 16 Hz, H-6), 2.13 (*br d*, J = 16 Hz, H-6), 1.81 (*m*, 10-Me).

[6,10-$^2H_2$]-*Deoxygeniposide* (d_2-**13**). Similarly **15a** (6.7 g) was treated with $^2HCO_2{}^2H$ (1 g) for 1.5 hr to give after chromatography (D-column) the deuterated glucoside (d_2-**13**, 2.65 g). ^1H NMR showed the presence of 0.4 ^2H in the C-6 position and 0.8 ^2H in the C-10 position.

[OMe-$^2H_3$]-*Deoxyloganin* (d_3-**8**). Deoxygeniposide (**13**, 760 mg) in EtOH (15 ml) was hydrogenated over Pd/C (300 mg) until 1 mol of H_2 had been absorbed (*ca* 1 hr). The catalyst was filtered off and evapn followed by RP-chromatography (H_2O–MeOH, 1.5:1 and 1:1, Lobar, C-column) gave 650 mg of crude **8** (HPLC at this point showed that *ca* 20% of the C-8-epimer was present. Saponification was effected with 1 M NaOH (7 ml) for 2 hr at 70°. The mixture was neutralized with IR-120 (H^+-form), HOAc was added (0.5 ml) and the product chromatographed as above (H_2O–MeOH, 1.5:1) to yield crude deoxyloganic acid (7, 525 mg). This was evapd twice with ^2H_2O (1.5 ml), dissolved in EtO^2H (10 ml) and treated with an excess of $^2H_2$–CH_2N_2 [16]. Chromatography as above followed by crystallization (EtOAc satd with H_2O) provided the deuterated glucoside (d_3-**8**, 437 mg) which still contained *ca* 20% of the C-8 8-epimer (HPLC). Application of this to the Hibar column (H_2O–MeOH, 1.5:1, 20 ml/min) in 45 mg portions gave pure d_3-**8** (335 mg) containing less than 1% of the C-8-epimer (HPLC). Crystallization (99% EtOH) gave 298 mg, mp 154–155°. ^1H NMR (500 MHz, D_2O): essentially as described [5]; integration showed a content of 93% $^2H_3$ in the methyl ester group.

[6,7,8,10-$^2H_4$]-*Deoxyloganin* (d_4-**8**). Sodium (1.4 g) was reacted with ^2H_2O (5 ml) and the evolved $^2H_2$ collected in a polyethylene bag. d_2-**13** (1.54 g) was evapd twice with ^2H_2O

(1.5 ml) and dissolved in EtO^2H (15 ml). Pd/C (500 mg) was added and hydrogenation was performed with the above gas after displacement twice with both N_2 and $^2H_2$. After chromatography d_4-**8** (1.05 g) was isolated as a foam. HPLC showed that *ca* 20% of the C-8-epimer was present. A deuterium content of *ca* 2 was determined at 10-Me (broad signal at δ1.05) by ^1H NMR (90 MHz, D_2O).

11-*Hydroxy*-[6,7,8,10-$^2H_4$]-*iridodial glucoside* (d_4-**16**). Acetylation of d_4-**8** (1.05 g) gave the tetraacetate (d_4-**8a**, 1.42 g). Reduction of this was performed essentially as described [10]. Thus, under N_2, $LiAlH_4$ (1.6 g) was suspended in dry THF (45 ml) and MeOH (4.05 ml) was added, with stirring, from a syringe during 15 min at -20 to $-40°$. After stirring for 2 hr and allowing the temp to rise to 5°, the mixture was cooled to $-60°$ and the above tetraacetate in THF (20 ml) added during 15 min. The temperature was allowed to rise to 5° and stirring was contd for 2 hr. Work-up as described [10] gave a foam (1.1 g) which was chromatographed (C-column, H_2O–MeOH, 3:2; detection at 206 nm) to yield d_4-**16** (455 mg, 48%) as a foam. ^1H NMR (90 MHz, D_2O): δ6.33 (*br s*; H-3), 5.31 (*d*, J = 3.5 Hz, H-1); 4.20 and 3.98 (AB-system, J = 12 Hz, 11-CH_2); a signal at *ca* 20% intensity at δ5.47 (*d*, J = 1.5 Hz, H-1) showed the presence of the C-8-epimer.

[6,7,8,10-$^2H_4$]-*Iridotrial glucoside* (d_4-**6**). PtO_2 (20 mg) was suspended in H_2O (1.5 ml) and reduced with H_2 for 1 hr. The labelled 11-hydroxyiridodial glucoside (d_4-**16**, 135 mg) dissolved in H_2O (5 ml) was added and stirring under O_2 was performed for 5.5 hr, when HPLC (detection 219 nm) showed the reaction to be complete. Filtering and evapn gave a syrup (135 mg) which was purified by chromatography (B-column; H_2O–MeOH 3:2) to give the 'pure' glucoside (d_4-**6**, 131 mg, 96%) as a foam. ^1H NMR (90 MHz, D_2O): δ9.13 (*s*, H-11), 7.48 (*br s*, H-3), 5.57 (*d*, J = 4 Hz, H-1); as above, a signal of *ca* 20% intensity at δ5.73 (*d*, J = 3.5 Hz, H-1) showed the presence of the C-8-epimer.

[6,7,8,10-$^2H_4$]-*Iridodial glucoside* (d_4-**5**). Acetylation of the labelled 11-hydroxyiridodial glucoside (d_4-**16**, 250 mg) gave the pentaacetate (400 mg) which was dissolved in EtOH (30 ml). Pd/C (250 mg) and NEt_3–HCO_2H complex (TEAF [6], 105 mg, corresponding to 1.2 meq HCOOH) was added and the mixture was refluxed under stirring for 1 hr when TLC showed the reaction to be complete. The catalyst was filtered off and the solution concd *in vacuo* followed by evapn twice with toluene (20 ml). The residue was dissolved in MeOH and deacetylated with NaOMe for 20 min when HOAc in excess was added. Evaporation and chromatography (B-column, 1:1) gave the glucoside (d_4-**5**, 165 mg, 69%). ^1H NMR (500 MHz, D_2O): δ6.03 (1H, *br s*, H-3), 5.23 (1H, *d*, J = 3.5 Hz, H-1), 3.95–3.25 (6H, H-2′ to H-6′), 2.56 (1H, *t*-like, H-5), 1.97 (0.6 H, *m*, H-6), 1.86 (0.4H, *m*, H-7 and H-8), 1.80 (1H, *dd*-like, H-9), 1.55 (3H, *s*, 11-Me), 1.48 (0.7H, *m*, H-6), 1.20 (0.7H, *m*, H-7), 1.04 (1.0H, *m*, 10-Me); the product was contaminated with the C-8-epimer (20%) determined from the intensity of the peak at 5.39 (*d*, J = 2 Hz, H-1). Due to the presence of this impurity the accuracy of the above integrals was limited to *ca* 0.1 H.

[6,7,8,10-$^2H_4$]-*Iridotrial* (d_4-**4**). The glucoside (d_4-**6**, 36 mg) was dissolved in 0.1 M NaOAc buffer (5 ml) and β-glucosidase (Sigma, 5 mg, 5.7 units) was added. After stirring overnight at 30° the mixture was extracted with Et_2O (3 × 5 ml). The extract was dried (Na_2SO_4) and the solvent evapd under N_2 to give d_4-**4** as an oil (13 mg) which was used for the feeding experiments.

[6,7,8,10-$^2H_4$]-*Iridodial* (d_4-**3**). The glucoside (d_4-**5**, 27 mg) was treated as above to give the aglucone (8 mg).

[3,11-^{13}C]-*Iridotrial glucoside* (c-**6**). The preparation of this compound has been described elsewhere [9]. The compound contained 86% ^{13}C as C-11 and 5% as C-3.

[3,11-^{13}C]-*Iridodial glucoside* (c-**5**). Labelled iridotrial glucoside (*c*-**6**, 115 mg) was dissolved in MeOH (20 ml) and $NaBH_4$ (60 mg) was added under stirring. After 15 min the reaction was complete (HPLC, 219 nm), excess OAcH was added, and the solvent removed. The residue was acetylated (2 ml pyridine, 1 ml Ac_2O, 2 hr) to give the pentaacetate (*c*-**16a**, 175 mg). This was hydrogenated in EtOH with TEAF–Pd/C, deacetylated and purified by chromatography as above to give pure *c*-**5** (80 mg, 73%) as a crystallizing glass. ^1H NMR (90 MHz, D_2O): δ6.02 (*m*, H-3), 5.22 (*d*, J = 3.5 Hz, H-1), 2.5 (*m*, H-5), 1.54 (*d*, $J_{H\text{-}11,C\text{-}11}$ = 126 Hz, 11-CH_3), 1.04 (*d*, J = 6 Hz, 10-CH_3). ^{13}C NMR (22.6 MHz, D_2O): δ132.9 (C-3), 16.0 (C-11)-relative intensities 1:13.

Deoxyloganic acid (**7**) *from iridotrial glucoside* (**6**). Iridotrial glucoside (= 5-deoxystansioside), isolated from *Chaenostoma foetidum* (0.3% of fr. wt), was acetylated to give the tetraacetate. To a soln of this (**6a**, (1.52 g) in *t*-BuOH (35 ml) and 2-methyl-2-butene (9 ml) was added a soln of $NaClO_2$ (3 g, 40%) and NaH_2PO_4 (2 g) in H_2O (30 ml) under vigorous stirring [11]. After 16 hr an additional portion of the latter soln was added and stirring contd for 16 hr more. At this point the reaction was complete (checked by TLC), and excess oxidant was destroyed by $K_2S_2O_6$ at 0°. The cold mixture was extracted with CH_2Cl_2 (3 × 50 ml) and the organic phase with H_2O (2 × 100 ml). The organic phase was dried and the solvent removed. Deacetylation followed by chromatography (C-column, 2:1) gave pure **7** (636 mg, 64%), crystallized from EtOH, mp 113–115°; $[\alpha]_D^{20}$ −85° (MeOH; *c* 0.9). Reported [17]: mp 113–115°). ^1H NMR (500 MHz, D_2O): δ 7.42 (*d*, J = 0.9 Hz, H-3), 5.30 (*d*, J = 4.3 Hz, H-1), 2.84 (*br q*, J = 7.3 Hz, H-5), 2.10 (*ddt*, J = 4, 13 and 8 Hz, H-6β), 1.84 (2H, H-7β and H-8), 1.78 (*m*, H-9), 1.37 (*br dq*, J = 13 and 7 Hz, H-6α), 1.17 (*dq*, J = 12 and 8 Hz, H-7α), 1.11 (*d*, J = 6.5 Hz, 10-Me). ^{13}C NMR (125 MHz, D_2O): δ172.1 (C-11), 152.6 (C-3), 113.0 (C-4), 97.9 (C-1), 48.5 (C-9), 35.7 (C-8), 33.5 (C-5), 33.2 (C-7), 32.1 (C-6), 20.1 (C-10) and 99.6, 73.5, 76.5, 70.3, 77.1, 61.5 (C-1′ through C-6′). Acetylation provided the tetraacetate (**7a**), mp (EtOH) 194–195°; $[\alpha]_D^{20}$ −86° (EtOH; *c* 0.8). Reported [18]: mp 184–186°; $[\alpha]_D^{18}$ −89° (EtOH; *c* 0.8). ^1H NMR (90 MHz, $CDCl_3$): δ7.4 (*d*, J = 1 Hz, H-3), 2.8 (*m*, H-5), 2.10, 2.03, 2.00, 1.95 (*s's*, 4 × AcO), 1.07 (*d*, J = 5 Hz, 10-Me).

Deoxyloganic acid aglucone (**19**) wsas prepared from **7** (32 mg) by treatment with β-glucosidase as above to give the product (18 mg) characterized by ^1H NMR (90 MHz, $CDCl_3$): δ7.48 (*d*, J = 1 Hz, H-3), 4.88 (*d*, J = 7 Hz, H-1), 2.82 (*br q*, J = 7.5 Hz, H-5), 1.06 (*d*, J = 6 Hz, 10-Me). Acetylation provided the monoacetate (**19a**); ^1H NMR (90 MHz, $CDCl_3$): δ7.22 (*br s*, H-3), 6.30 (*d*, J = 3 Hz, H-1), 3.06 (*m*, H-5), 2.05 (*s*, AcO), 1.05 (*d*, J = 5.5 Hz, 10-Me).

[3,11-^{13}C]-*Deoxyloganic acid* (c-**7**) was prepared as the unlabelled compound above from *c*-**6a** and isolated as a foam. ^1H NMR (90 MHz, D_2O): δ7.46 (*dd*, $J_{3,5}$ = 1 Hz and $J_{C\text{-}11,H\text{-}3}$ = 3.5 Hz) the remaining part being unchanged from the unlabelled compound. ^{13}C NMR (22.6 MHz, D_2O): δ172.3 (C-11), 152.5 (C-3), 113.0 (*d*, J = 75 Hz, C-4); when comparing with a spectrum of the unlabelled compound the enrichments were calculated to 5% for C-3 and 87% for C-11 comparing well with a labelling equal to that found for *c*-**5** and *c*-**6**.

[3,11-^{13}C]-*Deoxyloganic acid aglucone* (c-**19**) was prepared from the glucoside (*c*-**7**, 25 mg) as above to give the product (12.5 mg).

(*α-Carboethoxyethylidene*)-*triphenylphosphorane* (**20**). Methyl iodide (1 g) was added to a soln of carboxymethylene-triphenylphosphorane (2.32 g) in dry EtOAc (20 ml) under N_2 in a screwcap vessel [19]. The mixture was stirred overnight at 78°, filtered and the solid dissolved in MeOH (10 ml). H_2O (40 ml) was added to give a clear soln. Adjustment to pH 9 with 1 M NaOH was followed by extraction with CH_2Cl_2 (2 × 10 ml).

The extract was dried (Na_2SO_4) and diluted to 25.0 ml to give a stock soln. Evaporation of 0.5 ml gave 52 mg (107%!) of a solid; ^{1}H NMR was essentially as described [14], but with some impurities present.

[^{13}C-*Methyl*]-(α-*carboethoxyethylidene*)-*triphenylphosphorane* (c-**20**) was prepared from ^{13}C-methyl iodide (Stohler 99% ^{13}C) as the unlabelled compound to give a stock soln (25 ml) as above.

[9-^{13}C]-10-*Hydroxy-geraniol* (c-**17**). (E)-6-Acetoxy-4-methyl-4-hexenal (575 mg, prepared by ozonolysis of geranyl acetate) was dissolved in half (12.5 ml) of the the above soln of *c*-**20** in a screwcap vessel and stirred under N_2 for 36 hr. Work-up followed by reduction with $LiAlH_4$–$AlCl_3$ essentially as described [14] gave the labelled compound (*c*-**17**, 220 mg; a 37% utilisation of MeI). ^{1}H NMR (90 MHz, $CDCl_3$) was essentially as reported [14], but the ^{13}C NMR spectrum (22.6 MHz) showed enhanced peaks at δ21.0 and 13.5 (3:17)—see comments in the Results.

(3S)-[9-^{13}C]-10-*Hydroxy-citronellol* (c-**18**) was prepared from (4*S*)-6-acetoxy-4-methylhexanal (575 mg, made by ozonolysis of (3*S*)-citronellol) as the preceding compound, to give *c*-**18** (236 mg; a 39% utilisation of MeI). Again the ^{13}C NMR spectrum showed two enhanced peaks at δ21.1 and 13.5 (3:17)—see Results.

[2-^{13}C]-*Mevalonic acid* (*c*-**1**) as the K-salt was prepared from the corresponding mevalonolactone (100 mg; ICN Biomedicals, 99% ^{13}C) by dissolution in 1 M KOH (7.6 ml), and this soln was used for the experiments.

Work-up of plant material. Iridoids in *Verbena officinalis* were isolated by extraction of a large portion (1 kg) of fresh, young (10–25 cm, just before flowering) plants with EtOH (2 × 2.5 l). The concd extract was partitioned between H_2O and Et_2O. The aq. fraction was passed through Al_2O_3 (750 g), washed with H_2O (2 l), and evapd to dryness. The residue was triturated with MeOH and passed through active C to give a colourless product. Chromatography (D-column, 4:1, 2 portions) with AcOH added (5 ml, in order to ascertain retention of terpenoid carboxylic acids) gave a very polar fraction (mainly carbohydrates) which was discarded, followed by crude cornin (**11**, 5.0 g, 0.5%). From this was crystallized compound **11** (EtOH, 3.3 g); ^{13}C NMR spectrum (125 MHz, D_2O): δ220.4 (C-6), 169.9 (C-11), 154.3 (C-3), 104.4 (C-4), 97.0 (C-1), 52.9 (OCH_3), 44.9 (C-9), 43.7 (C-7), 43.4 (C-5), 29.6 (C-8), 19.8 (C-10), 99.9, 77.1, 76.4, 73.4, 70.3 and 61.4 (C-1′ to C-6′). The mother liquors were shown to contain *ca* 3% hastatoside (**12**, *ca* 50 mg, 0.005%) by HPLC. The remaining compounds (1.5 g) were eluted from the column with MeOH and rechromatographed (C-column, 3:1, to 1:1) giving first dihydrocornin (**10**, 100 mg, 0.01%); ^{13}C NMR spectrum (125 MHz, D_2O): δ170.8 (C-11), 153.0 (C-3), 110.1 (C-4), 96.6 (C-1), 77.8 (C-6), 52.7 (OCH_3), 47.1 (C-9), 41.8 (C-7), 41.7 (C-5), 33.8 (C-8), 19.8 (C-10), 99.4, 77.1, 76.4, 73.5, 70.4 and 61.5 (C-1′ to C-6′). Then came a fraction containing deoxyloganic acid (**7**, 20 mg). Methylation (CH_2N_2) followed by rechromatography (A-column, 1:1) gave almost pure deoxyloganin (**8**, 5 mg, 0.0005%), identified by ^{1}H NMR. In the remaining fractions from the above C-column, neither deoxyloganin (**8**) nor dihydrocorninic acid (**9**) could be detected.

Feeding experiments. Glucosides were dissolved in H_2O (5 ml) and stems (1–3, *ca* 10 g, freshly cut under H_2O) were immersed in this soln. Usually, the precursor was absorbed within less than 12 hr and more H_2O was then added in order to sustain the plants for the specified period.

In order to emulsify non-glucosidic precursors Tween 80 is often used. We tested this emulsifier as well as others in concentrations of 1 and 2 mg/ml, but except for dish-washing detergent (according to ^{1}H NMR a long chain aliphatic sulphate) at 2 mg/ml, all proved detrimental to the plants. Furthermore, the detergent was able to emulsify the precursors tested. Thus, these were stirred with 5 ml of the above soln before being fed to plants.

Work-up of feeding experiments. The plant stems (1–3, *ca* 10 g) fed with precursor were blended with EtOH (3 × 50 ml), partitioned between H_2O and Et_2O, and the aq. fraction passed through Al_2O_3 (25 g) followed by elution with H_2O (150 ml). Evaporation gave the crude extract (0.2–0.4 g). Application to a B-column (3:1) provided chromatographically pure cornin (**11**). After eluting the column with MeOH, dihydrocornin (**10**) and other, minor components were isolated on an A-column (2:1). Due to the small quantities of **10** isolated, the exact amounts were determined by HPLC comparing with a soln of known concentration. Usually, only **10** and **11** were detected and isolated, but in a few cases other compounds were present. These cases are described below.

Experiment 8 (Table 2). 11-Hydroxy-iridodial glucoside (d_4-**16**, 4 mg, 33%) was isolated. The ^{1}H NMR spectrum was identical to that of the preparation above.

Experiment 11 (Table 3). Two additional fractions of a few mg's each were obtained, both derived from the labelled 10-hydroxycitronellol. According to the NMR spectra, the first consisted of 2,6-dimethyl-8-hydroxy-oct-2-enoic acid 1-β-glucopyranosyl ester, while the other was a mixture of the two β-glucopyranosides of *c*-**18**.

Experiment 14. Labelled iridodial glucoside (c-**5**, 4.9 mg, 24%) was isolated and identified by NMR.

Experiment 15. Labelled precursor (*c*-**6**, 1.7 mg, 14%) and 11-hydroxy-iridodial glucoside (*c*-**16**, 7.2 mg, 58%) were isolated and identified by NMR.

Acknowledgements—We thank the staff of The Botanical Garden of The University of Copenhagen for growing the plant material. Access to NMR facilities was provided by the Danish Natural Science Council and the Carlsberg Foundation.

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