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THE EFFECT OF METABOLIC PERIOD, DOSE AND APPLICATION METHOD ON THE INCORPORATION OF DEOXYLOGANIN INTO CORNIN IN *VERBENA OFFICINALIS*

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Key Word Index—*Verbena officinalis*; Verbenaceae; biosynthesis; ^{2}H NMR; experimental parameters: deoxyloganin; cornin; dihydrocornin; iridodial glucoside.

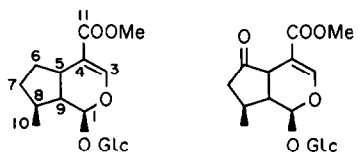
Abstract—The incorporation of [methoxy-^{2}H]deoxyloganin into cornin in *Verbena officinalis* has been investigated. With wick feeding, maximal incorporation (15%) was obtained when the applied dose was 1.3 μmol/g plant and the metabolic period was 7 days. Feeding of an aqueous solution of the precursor (3.6 μmol/g plant, 7 days) to cut stems gave 14% incorporation. The incorporations have been determined by ^{2}H NMR spectroscopy. Dihydrocornin has not previously been reported from *Verbena*. Here, however, it was isolated in all cases with varying degrees of deuterium labelling. We have shown that dihydrocornin is an intermediate in the biosynthesis of cornin from deoxyloganin. Finally, it was shown that iridodial glucoside is incorporated (0.7%) into cornin in *V. officinalis*.

INTRODUCTION

The application of ^{2}H NMR in the study of biosynthetic pathways is a comparatively novel technique [1]. The method offers several advantages compared to radioisotope investigations [1, 2] but has a disadvantage in its smaller sensitivity. It is, therefore, important to establish the conditions for which the incorporation of a given precursor is maximal. The effect of applied dose and metabolic period on the degree of incorporation and dilution value has previously been subject to a comprehensive study [3]. The formation of quercetin in *Fagopyrum tataricum* from several precursors was investigated. At low doses, the dilution values varied a great deal, whereas the degree of incorporation remained relatively constant over a large dose interval. The degree of incorporation of L-phenylalanine into quercetin was constant at metabolic periods of 8–24 hr, whereas smaller values were obtained at shorter and longer metabolic periods.

To us, however, it was important to know whether the same conclusions could be reached with an advanced precursor. We have previously studied the conversion of deoxyloganin (**1**) to cornin (**2**) in *Verbena officinalis* [2, 4] and have, therefore, chosen this as a model system for an investigation of the effect of dose, metabolic period and application method on the degree of incorporation and dilution value.

In an earlier paper [2], we have shown that dihydrocornin (**3**) is a precursor for **2**. Here we present evidence that **3** is also an intermediate in the biosynthesis of **2** from **1**.



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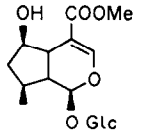

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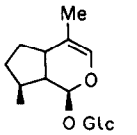

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*In the numbering of the compounds the letter **a** denotes a fully acetylated compound, and the letter **d** denotes a compound deuterated as specified in the text.

RESULTS AND DISCUSSION

[methoxy-^{2}H]Deoxyloganin (**1d**)* was prepared by treatment of deoxyloganic acid with deuterated diazomethane [2]. We have earlier shown [2, 4] that the methoxycarbonyl group of **1** is preserved during the conversion of **1** to cornin (**2**). The precursor **1d** was fed as an aqueous solution to *V. officinalis* either by the cotton wick method or by letting the cut stem suck the solution up. After the experiment, the glucosides were isolated and the iridoids were separated by reversed phase chromatography. Cornin (**2**) was acetylated to give **2a** which was recrystallized from ethanol and the degree of incorporation as well as the dilution value were estimated by ^{2}H NMR [2]. In all the experiments small amounts of dihydrocornin (**3**) and deoxyloganin (**1**) were isolated. The deuterium contents of these compounds were estimated by mass spectrometry. The results (presented in Table 1) show that the incorporation of **1** into **2** in *V. officinalis* is efficient in the dose range of 0.5–5 μmol/g plant, with a maximum at 1.3 μmol/g plant. At very high doses the incorporation decreases significantly. As the speed of ^{2}H NMR measurements, which are used to estimate the degree of incorporation, grows with increasing absolute amount of deuterium, it would from that point of view be optimal to use doses up to 5 μmol/g plant. The method of

Table 1. Incorporation of 1 into 2 and 3 in *V. officinalis*

	Method of application					
	Cotton wick					Cut stem
Wt of plant material (g)	34.4	21.2	23.2	3.2	2.8	15.7
Amount of **1d** fed (mg)	7.9	10.7	30.5	25.2	32.6	24.0
Dose (μmol/g plant)	0.61	1.3	3.5	21	31	4.8
Compounds isolated						
1 (mg)*	0.32	0.30	1.7	1.2	1.3	1.6
Enrichment (%)	89	87	92	92	92	94
3 (mg)*	4.9	4.2	6.8	1.9	3.1	7.5
Enrichment (%)	29	47	67	84	83	76
2 (mg)	297	165	181	15	17	149
Incorporation (%)	8.3	15	11	1.7	1.5	14
Dilution value	435	102	53	33	34	44

Degree of incorporation and dilution value with varying dose and application method. Metabolic period 7 days.

*Measured by HPLC. See Experimental.

Table 2. Incorporation of 1 into 2 and 3 in *V. officinalis*

	Metabolic period (days)			
	1.8	3	7	30
Wt of plant material (g)	19.7	16.6	23.2	17.5
Amount of **1d** fed (mg)	18.6	20.6	30.5	23.3
(μmol/g plant)	2.5	3.3	3.5	3.6
Compounds isolated				
1 (mg)	3.8	4.4	1.7	0.4
Enrichment (%)	92	94	92	92
3 (mg)	5.4	4.6	6.8	2.3
Enrichment (%)	18	41	67	55
2 (mg)	222	159	181	142
Incorporation	3.1	5.5	11	11
Dilution value	370	135	53	56

Degree of incorporation and dilution value as a function of metabolic period.

application, however, seems to have a limited effect on the degree of incorporation, as the incorporation in the cut stem experiment is of the same order of magnitude as the incorporation from the cotton wick experiments with the corresponding doses.

An unexpected feature of these experiments was that dihydrocornin (**3**) was isolated. Dihydrocornin (**3**) has not previously been isolated from *Verbena* but it co-occurs with cornin (**2**) in *Cornus* [5]. In the present series of experiments, however, it was isolated in all cases. Mass spectrometry showed that it was partly deuterium labelled with an enrichment ranging from 18 to 84%. The fact that **1** is converted to **3** combined with the fact that **3** is a precursor for **2** [2] shows that dihydrocornin (**3**) is an intermediate in the biosynthesis of cornin (**2**).

The intermediacy of deoxyloganin (**1**) is, however, more dubious. In all cases deoxyloganin was isolated but the enrichment was nearly equal to that of the precursor fed (94.2%). A possible explanation for this could be that there is no *de novo* synthesis of **2** during the flowering period. Furthermore, it has not been possible to detect **1** in *V. officinalis* by HPLC. In a small plant tested in this way, 0.5–1% of **2** and 0.01% of **3** was detected, while no **1** was observed with a detection limit of 5 ppm. The intermediacy of **1** in the biosynthesis of **2** must, therefore, still be considered uncertain.

Finally, iridodial glucoside (**4**) was tested as a precursor for cornin (**2**). Deuterium labelled iridodial glucoside (**4d**) was made in the following way. [7,8,10-^{2}H]Deoxyloganin [2] was reduced with sodium borohydride [6] to a mixture of deuterium labelled deoxyloganic acid and 11-hydroxy iridodial glucoside. The latter was acetylated and reduced with a triethylamine–formic acid complex and palladium on carbon to give **4ad**. Deacetylation gave **4d** which was crystallized from water–methanol. When fed to *V. officinalis* **4d** was incorporated into cornin (**2**). The ^{2}H NMR spectrum of **2a** thus obtained was identical to that discussed earlier [2, 4] (except that the methoxycarbonyl group was not labelled). The incorporation was estimated as 0.7%, considerably less than the incorporation of **1** into **2**. Again, deuterium labelled dihydrocornin was isolated.

EXPERIMENTAL

^{2}H NMR spectra were recorded as described in ref. [2]. [methoxy-^{2}H]Deoxyloganin (**1d**) was prepared from deoxy-

loganic acid and $C^2H_2N_2$ as described in ref. [2]; mp 150–152°; $[\alpha]_D^{20}$ −90° (MeOH; *c* 0.46). The content of 8-epi-deoxyloganin was determined by HPLC to be 10%. The MS showed a distribution of label of $^2H_1$, 2%; $^2H_2$, 13.5%; and $^2H_3$, 84.5% (calculated from *m/z* 212–215) with a mean of 2.83 ^2H/mol (enrichment 94.2%).

General procedure for the administration of labelled precursors and for the isolation of iridoids. The precursor was administered as an aq. soln during the flowering period of the plants in August. After harvesting, the plants were stored in polyethylene bags at −23° until work-up. The frozen plant material was extracted twice with EtOH, evaporated, dissolved in H_2O and extracted with Et_2O to remove fats, etc. The aq. soln was filtered through a column of neutral Al_2O_3 followed by washing with H_2O. The eluate was concd and treated with activated C. The isolation of the iridoids was accomplished by reversed phase chromatography (RP-8) with H_2O–MeOH (3:1 and 2:1) as the eluent.

Cornin was acetylated and recrystallized for ^2H NMR, while the amount and purity of dihydrocornin (**3**) and deoxyloganin (**1**) was determined by HPLC using standard solns. The enrichments of **1** and **3** were determined by MS at 70 eV.

Preparation of [7,8,10-^2H]*iridodial glucoside* (**4d**) *from* [7,8,10-^2H]*deoxyloganin.* [7,8,10-^2H]Deoxyloganin (715 mg) containing *ca* 20% [7,8,10-^2H]-8-epi-deoxyloganin (prepared as described in ref. [2]) and 1.54 g $NaBH_4$ was dissolved in 15 ml H_2O. After heating the soln for 4 hr at 70–80° excess HOAc was added and the solvent evaporated. The residue was subjected to reversed phase chromatography (RP-18) using H_2O–MeOH (4:1) as the eluent to give [7,8,10-^2H]deoxyloganic acid (450 mg) and 11-hydroxy-[7,8,10-^2H]iridodial glucoside. The latter was acetylated (255 mg) and mixed with a similar preparation to give 655 mg, (1.17 mmol), which was dissolved in EtOH (30 ml). TEAF ($5HCOOH \cdot 2NEt_3$, 0.65 mmol) and Pd–C (5%, 200 mg) were added. The soln was refluxed under N_2 and the progress of the reaction followed by TLC. After 30 min a further amount of Pd–C (5%, 100 mg) was added, and 20 min later TEAF (0.10 mmol) was added. After 70 min the reaction was complete (TLC) and the catalyst was removed. (In a similar preparation the reaction was complete in 20 min.) Evaporation gave crude **4ad** (596 mg), which was deacetylated with NaOMe in MeOH. Neutralization with HOAc and evaporation of the solvent gave crude **4d** which was crystallized from H_2O–MeOH (5:1) to give colourless crystals of **4d** (240 mg) containing 3% [7,8,10-^2H]-8-epi-iridodial glucoside (HPLC). The physical data for **4d**: mp 159–160°, $[\alpha]_D$ −120° (MeOH; *c* 0.29) are in reasonable agreement with those reported in ref. [8] [mp 168–169°, $[\alpha]_D$ −110.3° (MeOH; *c* 0.46)] and **4d** was not further purified. The MS showed that **4d** contained 5% $^2H_1$, 10% $^2H_2$, 16% $^2H_3$, 19% $^2H_4$, 21% $^2H_5$, 16% $^2H_6$, 9% $^2H_7$ and 3% $^2H_8$ (calculated from *m/z* 168–178) with a mean of 4.45 ^2H/mol.

Administration of [7,8,10-^2H]*iridodial glucoside* (**4d**) *to* V. officinalis *and isolation of iridoids.* [7,8,10-^2H]Iridodial glucoside (**4d**) (25.1 mg) was administered as an aq. soln by the cotton wick method to two *V. officinalis* (14.0 g) plants during the flowering period in September. After a metabolic period of 7 days the plants were worked-up as described above. Cornin (**2**) (68 mg) and dihydrocornin (**3**) (0.8 mg) as well as a small amount of **4d** were isolated. The reisolated **4d** contained the same activity (MS) as the fed material.

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