Phytochemistry, Vol. 27, No. 2, pp. 391–395, 1988.
Printed in Great Britain.

0031-9422/88 $3.00+0.00
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INTERMEDIATES IN THE FORMATION OF PUBERULIN BY *AGATHOSMA PUBERULA*

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(*Received* 23 *June* 1987)

Key Word Index—*Agathosma puberula*; Rutaceae; biosynthesis; coumarins; puberulin.

Abstract—Direct testing of ^{14}C-labelled aesculetin has yielded results in accord with its previously postulated role as the intermediate between umbelliferone and scopoletin in the biosynthesis of puberulin by *A. puberula*. Mass spectrometric analysis of an acidic fraction from this species, which should contain all its free phenolic coumarins, indicated the presence of isofraxidin and fraxetin, which was confirmed by GC. [8-*O*-^{14}Me] Isofraxidin was, on average, utilized comparably to [^{14}Me] scopoletin in puberulin formation, and in two experiments the incorporations of > 1% were among the best that have been observed for any puberulin precursor. Participation of isofraxidin strongly implicates fraxetin as an intermediate. The sequence: *p*-coumaric acid→→umbelliferone→aesculetin→scopoletin→fraxetin→isofraxidin→puberulin is proposed as a feasible biosynthetic route.

INTRODUCTION

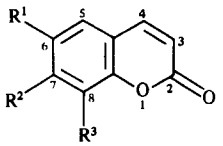
Earlier studies in these laboratories have addressed the question of the elaboration of hydroxylation patterns in simple coumarins. Both in the case of 6,7-dihydroxycoumarin (aesculetin, **1a**) in *Cichorium intybus* [1] and of 7,8-dihydroxycoumarin (daphnetin, **1b**) in *Daphne mezereum* [2], radiotracer investigations with carbon-14 have strongly indicated their derivation by hydroxylation of 7-hydroxycoumarin (umbelliferone, **1c**).

A more complex pathway leads to puberulin (**1d**), the 7-*O*-prenyl ether of isofraxidin (**1e**), which also derives from umbelliferone in *Agathosma puberula* Fourc. [3]. This conversion involves two further hydroxylations, two *O*-methylations and an *O*-prenylation. The earlier ^{14}C tracer experiments showed generally good conversions of scopoletin (**1f**) to puberulin, and on this basis three of us [3] have proposed that 6-hydroxylation of umbelliferone first occurs, yielding aesculetin, followed by 6-*O*-methylation to scopoletin. Ferulic (3′-methoxy-4′-hydroxycinnamic) acid, a precursor of scopoletin in tobacco [4], was not well converted to scopoletin in *A. puberula*, and it has been suggested that the pathway in tobacco is exceptional [5].

In the investigations reported here we have tested aesculetin as a puberulin precursor in *A. puberula*, and studied the biosynthetic pathway beyond scopoletin, with the aid of mass spectrometric analyses of plant extracts and further tracer experiments.

RESULTS AND DISCUSSION

The partial biosynthetic sequence suggested in the earlier paper [3], involving the conversion of umbelliferone to scopoletin, implies the intermediacy of aesculetin. Aesculetin was not tested as a puberulin precursor during the earlier studies, but labelled aesculetin has now been synthesized and compared as a precursor to scopoletin and umbelliferone, which had both been shown to be effective. In Table 1 are presented the results of these experiments. The dilution values of ^{14}C observed for scopoletin are of the same order as previously reported, and we reiterate that the relatively high dilutions recorded for even the most effective precursors in these experiments reflect the high levels of preformed puberulin in our experimental plants which often fell in the range of 9–14 mg/g fresh weight. On average, the values for aesculetin are a little higher, a finding fully in accord with the theory that it is the immediate precursor of scopoletin. The Table also shows minimum percent incorporations. At the time these experiments were done serious lack of reproducibility of isofraxidin recoveries was being en-



1a $R^1 = R^2 = OH$
1b $R^2 = R^3 = OH$
1c $R^2 = OH$
1d $R^1 = R^3 = OMe$, $R^2 = O{-}CH_2{-}CH{=}CMe_2$
1e $R^1 = R^3 = OMe$, $R^2 = OH$
1f $R^1 = OMe$, $R^2 = OH$
1g $R^1 = OMe$, $R^2 = O{-}CH_2{-}CH{=}CMe_2$, $R^3 = OH$
1h $R^1 = OMe$, $R^2 = R^3 = OH$
1i $R^1 = OMe$, $R^2 = O{-}CH_2{-}CH{=}CMe_2$

(Positions not indicated are unsubstituted)

Abbreviations: CIMS, chemical ionization mass spectrum; EIMS, electron impact mass spectrum; TMC, 6,7,8-trimethoxycoumarin.

Table 1. Incorporation of ^{14}C from aesculetin and comparison substrates into the coumarin moiety of puberulin by *Agathosma puberula*

Substrate fed*	Specific activity of puberulin (Bq/mmol)	^{14}C Dilution	Weight of recovered isofraxidin (mg)	Minimum % incorporation
[3-^{14}C]Aesculetin	253	11500	18	0.032
	554	4860	53	0.28
[2-^{14}C]Umbelliferone	48	46000	19	> 0.01
	100	29000	60	> 0.01
[$^{14}CH_3$] Scopoletin	490	5000	8	0.012
	1060	5600	45	0.15

*Specific activities (MBq/mmol): aesculetin 2.60, scopoletin 5.95.
Date of feeding was 2,3 April 1984.

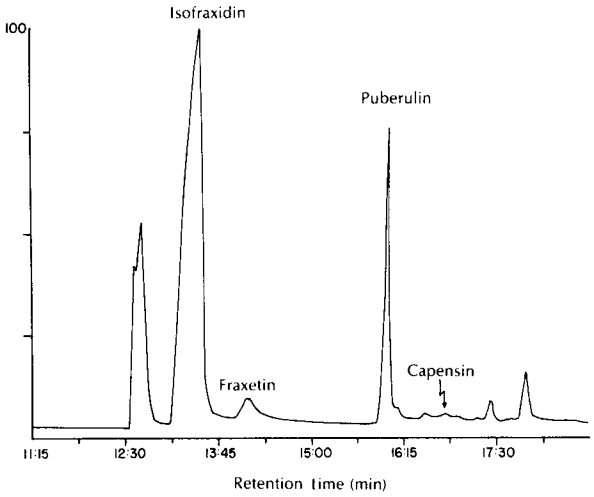


Fig. 1. Gas–liquid chromatogram of the acidic fraction from an extract of *A. puberula*.

countered, and we stress that the values are minimal; the true values in the first scopoletin feeding and the first two aesculetin feedings are almost certainly much higher. Nevertheless, the best incorporations of 0.15 and 0.28% are well above the accepted limit of significance [6]. We can offer no definite explanation of the failure of umbelliferone to approach the better levels of utilization noted earlier [3]. Instances of low incorporation were also observed in these earlier feedings, and we shall return to this phenomenon later in the discussion. Despite this anomaly the data for aesculetin and scopoletin are entirely consistent with the previously proposed sequence:

umbelliferone → aesculetin → scopoletin → → puberulin.

In the first paper on puberulin biosynthesis [3] three possible sequences of hydroxylation, *O*-methylation and *O*-prenylation were outlined which could lead from scopoletin to puberulin, involving four intermediates all known to occur naturally in some species: capensin (**1g**), fraxetin (**1h**), isofraxidin (**1e**), and scopoletin prenyl ether (**1i**). As all but the last of these contain free hydroxyl functions, they should be separable from any plant extract in which they are found by simple extraction into dilute alkali. Other acidic components would, of course, also be removed in this step.

An attempt was made to identify potential intermediates in the pathway from scopoletin to puberulin by trapping techniques, which involved administration of [2-^{14}C] umbelliferone simultaneously with inactive aesculetin, fraxetin, capensin, and isofraxidin, with metabolic periods of 24–72 hr before work-up. Acidic fractions of the plant extracts were then examined by TLC and HPLC, but no capensin or fraxetin could be detected. In one 72 hr experiment a TLC band corresponding to isofraxidin was present, but the recovered material was devoid of radioactivity. The puberulin samples recovered from these plants exhibited no consistent pattern of isotope incorporation permitting any inferences about intermediates.

The application of mass spectrometric analysis to the identification of components of complex mixtures of natural products, including plant extracts, is becoming well established [7, 8]. It therefore seemed possible that if there were even minute accumulations of intermediates from the scopoletin → → puberulin pathway in *A. puberula*, the high sensitivity of this technique might well permit their detection and tentative identification, as well as some degree of quantitative estimation. An ether-soluble, dilute alkali-extractable fraction was therefore examined with the aid of GC-MS. Standard samples of isofraxidin and fraxetin, two possible intermediates to be expected in this fraction, were used for comparative purposes.

The CH_4 CIMS of fraxetin consisted of $[M+1]^+$, *m/z* 209, as the base peak together with $[M+15]^+$, *m/z* 223, $[M+29]^+$, *m/z* 237, and $[M-15]^+$ *m/z* 193, each at 20%; *m/z* 61 (42%) and *m/z* 69 (40%). The CH_4 CIMS of isofraxidin consisted of $[M+1]^+$, *m/z* 223 as the base peak; $[M+15]^+$, *m/z* 237 (4%); $[M+29]^+$, *m/z* 251 (20%); $[M+41]^+$, *m/z* 263 (6%). The EIMS obtained at 70 eV of a crude acidic fraction from *A. puberula* (which should contain all the phenolic coumarins) consisted of *m/z* 222 as the base peak, *m/z* 236 (39%); *m/z* 247 and *m/z* 208 (30%); *m/z* 290 (26%); *m/z* 207 (22%); *m/z* 275 and *m/z* 193 (15%); *m/z* 276 and *m/z* 192 (13%); *m/z* 316 and *m/z* 179 (11%). Peaks at *m/z* 208 and *m/z* 222 suggest the presence of fraxetin and isofraxidin, and another at *m/z* 276 is consistent with the presence of capensin. In addition a peak at *m/z* 290 suggests puberulin, which, as a neutral compound, would not be expected in the acidic fraction. Because of instrumental difficulties, EI only was accessible

when the desorption probe was used with the crude acidic fraction, although CI would have been desirable in view of the intense $[M+1]^+$ peaks obtained with fraxetin and isofraxidin.

The mere presence of any of the potential intermediates in the extract does not, of course, establish its participation in the sequence of reactions leading to puberulin, since the possibility always exists that it is a metabolic end product. Nevertheless, evidence for the formation of such a compound in the plant implies that it is at least a potential intermediate, and justifies its further examination.

Of the two or possibly three potential intermediates demonstrated by mass spectrometry, isofraxidin was selected for further study, primarily because of the comparative ease of ^{14}C labelling. A supply of capensin was available and could be *O*-methylated with [^{14}C]MeI. Selective cleavage of the prenyl ether linkage of the resulting puberulin then yielded (8-*O*-^{14}Me] isofraxidin. With [^{14}Me] scopoletin as a comparison standard this labelled isofraxidin was tested as a puberulin precursor.

When processing the plants after administration of [^{14}C] isofraxidin one must ensure that any unmetabolized residues of this exogenous substance do not significantly contaminate the isofraxidin recovered after the hydrolysis of puberulin. In a blank experiment designed to assess the extent of such contamination, involving the addition of a known quantity of free [^{14}C] isofraxidin to a plant extract, over 99.98% of the added coumarin was removed in a subsequent purification, the residual counts being within normal counting error.

The results of the feeding experiments are given in Table 2. There is a distinct tendency to inconsistency of utilization. It is clear that in a number of feedings, especially the first three of the second scopoletin series, there was essentially no incorporation of the substrate label into puberulin, whereas in others the dilution approximated those observed in earlier studies. In the first series, in fact, the two dilution values for isofraxidin, and the second for scopoletin, are the lowest we have observed for any substrate in *A. puberula*. We cannot account for this extreme biological variability which, for scopoletin in this Table, amounts to a factor as much as 200, in that conditions of the experiment were maintained as constant as possible. It is, however, evident that, in the feedings where good incorporation did occur, isofraxidin is comparable as a puberulin precursor to scopoletin. If we reject the three extremely low scopoletin dilutions the average dilution for this substrate is 6160, compared to 8430 for isofraxidin. The wide scatter of the data would place more precise attempts at quantitation on an uncertain basis.

The methyl carbons of a sample of TMC, a derivative of the recovered isofraxidin from the third isofraxidin feeding of 4 February 1986 (Table 2), were isolated by demethylation. On the assumption that all the carbon-14 thus recovered originated from the 8-*O*-methyl carbon, its specific activity, corrected for dilution by the other two methyl carbons, was 94.4% of that of TMC, suggesting no significant randomization of the label into skeletal carbons.

At this stage of the research improvements in the isolation procedure described in the Experimental had been achieved, and reproducibility of recovery of the puberulin was much better than in the experiments of Table 1. The minimum per cent incorporation data of Table 2 can thus be regarded with more confidence, although actual weights present in the plants are still undoubtedly higher. As the values for the best two isofraxidin feedings were 1.0 and 1.3%, good utilization by accepted standards, there is no reason to doubt the role of this substrate as an effective precursor of puberulin in a fully functioning plant system.

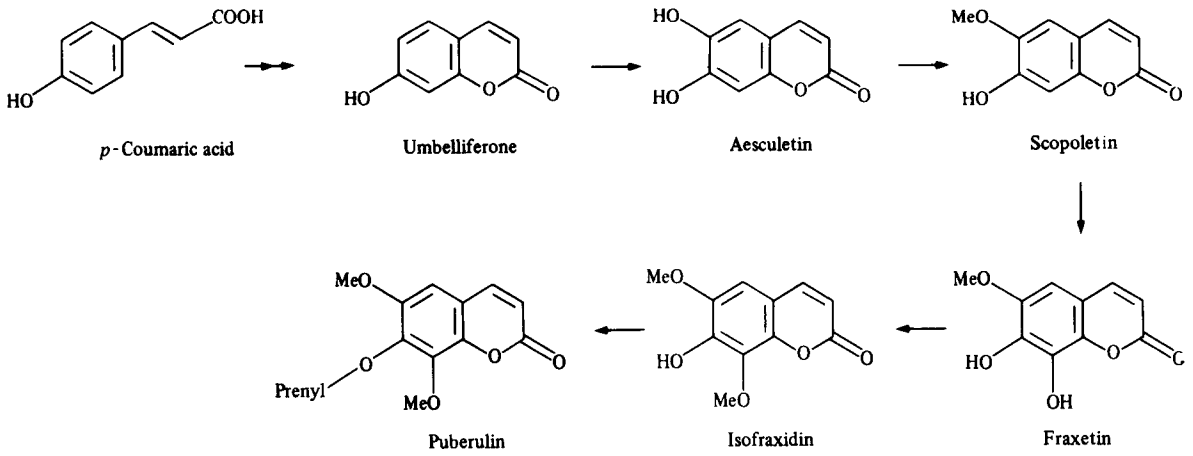
The indication from these experiments that isofraxidin approximates scopoletin in effectiveness for this biosynthetic pathway also strongly implicates fraxetin as the intermediate between these two coumarins. Existing evidence thus supports the route proposed in Scheme 1 for the formation of puberulin from 4′-hydroxycinnamic (*p*-coumaric) acid. All the precursors depicted here except fraxetin have been labelled with ^{14}C and their conversion to puberulin has been demonstrated *in vivo*. As mass spectrometry has identified isofraxidin and fraxetin as naturally present in *A. puberula*, these compounds fill the role of intermediates in the elaboration of puberulin by this species. The sequence of Scheme 1 is thus plausible as a biosynthetic pathway to puberulin.

We emphasize that the present findings in no way rule

Table 2. Incorporation of ^{14}C from isofraxidin and scopoletin into the coumarin moiety of puberulin by *Agathosma puberula*

Date of feeding	Substrate fed*	Specific activity of puberulin (Bq/mmol)	^{14}C Dilution	Weight of recovered isofraxidin (mg)	Minimum % incorporation
12 March 1985	[8-*O*-^{14}Me] Isofraxidin	10200	1030	63	1.3
		7120	1470	36	1.0
	[^{14}Me] Scopoletin	711	8370	65	0.20
		7800	763	21.5	1.1
28 February 1986	[8-*O*-^{14}Me] isofraxidin	515	11400	32	0.095
		423	13900	55	0.13
		1650	3560	87	0.60
		305	19200	69	0.11
	[^{14}Me] Scopoletin	143	59000	54	0.023
		59	140000	78	0.012
		54	160000	68	0.014
		902	9360	62	0.78

*Specific activities (MBq/mmol): isofraxidin 5.87, scopoletin 8.44.



Scheme 1.

out the possibility that other routes—via capensin, or even scopoletin prenyl ether for whose formation in this species there is no present evidence—lead to puberulin. A precedent exists for the biosynthesis of a polyalkoxycoumarin (isopimpinellin) via different sequences of hydroxylation and *O*-alkylation [9, 10], and there is no *prima facie* reason to disregard that possibility in the present case.

EXPERIMENTAL

Preparation of labelled substrates. The syntheses of [2-^{14}C] umbelliferone [11] and [^{14}Me] scopoletin [3] were described in earlier publications.

[3-^{14}C]Aesculetin. Dry pyridine (0.5 ml) was added to the vial in which a commerical sample of 3.7 MBq of [2-^{14}C]malonic acid had been placed and 2-hydroxy-4,5-dimethoxybenzaldehyde (182 mg, 1 mmol) [12] was dissolved in the resulting solution. Aniline (10 μl) was added and the reaction mixture was allowed to stand, stoppered, for 3 hr at room temp. Carrier malonic acid (220 mg) was added, dissolved with gentle warming, and the reaction allowed to proceed overnight. H_2O was then added and the solution acidified with HCl. After recovery by filtration the resulting ppt. of scoparone 3-carboxylic acid weighed 224 mg, 97% yield based on the aldehyde. Half of this product was demethylated by refluxing 0.5 hr with 57% HI (2 ml) containing a trace of red P, heated in a metal bath at 150°. After cooling, the reaction mixture was poured on ice and the resulting gelatinous, yellow ppt. of aesculetin 3-carboxylic acid was separated and washed repeatedly by centrifugation until the pH of the supernatant exceeded 5. The yield of dried product was *ca* 50%.

This was decarboxylated by modification of a published procedure [13]. It was deposited from Me_2CO as evenly as possible in the lower part of a side-arm test tube, a cold-finger condenser was inserted, the side arm plugged with glass wool, and the lower part of the tube immersed for 1 min in a metal bath at 300–305°. After cooling, all sublimed material was washed back into the tube with Me_2CO. A preliminary sublimation of the residue was done for 3 min at 170°, < 1 Torr, to remove traces of more volatile impurities; further sublimation at 220° then yielded aesculetin. This was recrystallized from H_2O (3.5 ml) to give 24.5 mg of product whose radiochemical purity was estimated after TLC in $CHCl_3$ MeOH, 24:1, to be 96%.

[^{14}Me] Isofraxidin. Capensin [14] **1g** (32 mg, 0.116 mmol), etc. anhydrous K_2CO_3 (0.10 g), [^{14}C]MeI (3.7 MBq) and dry Me_2CO (7 ml) were mixed at 0°. The solution was allowed to come to room temp. while being stirred for 22 hr. Carrier MeI (0.1 ml) and more dry K_2CO_3 (0.1 g) were added and stirring was continued for a further two days. H_2O (5 ml) was added and the Me_2CO removed *in vacuo*. Dilute NaOH (1 ml) and EtOAc (20 ml) were added, the mixture was shaken, and the EtOAc layer washed with H_2O (2 × 3 ml) and evapd. The residue was refluxed 1 hr with 4 ml of 2% H_2SO_4 in MeOH. H_2O (10 ml) was added and the MeOH removed *in vacuo*. The H_2O solution was extracted continuously with Et_2O for 1 hr. The Et_2O was evapd, the residue warmed with EtOAc (20 ml) and shaken with fresh $NaHCO_3$ solution (1 ml) and H_2O (2 ml), then dried with Na_2SO_4 and evapd. The isofraxidin (24 mg) was mixed with carrier isofraxidin (63 mg) and recrystallized from EtOAc to give 71 mg of [^{14}C] isofraxidin, 595 MBq/mmol.

Processing of plants. In the earlier investigations [3] unrecognized variables were noted which could lead to large variations in isofraxidin recoveries. Modifications in the isolation procedures have now overcome this difficulty to a large degree.

Trial experiments showed that when puberulin was refluxed for even 6 hr with MeOH more than 90% of the recovered material was phenolic, whereas with EtOAc it was recovered virtually unchanged after refluxing overnight (> 95% recovery). Accordingly, in the revised procedure, the plant was extracted in a Soxhlet apparatus 2 days with EtOAc instead of MeOH previously used. The extract (*ca* 80 ml) was washed with 2% NaOH (3 × 30 ml), then H_2O (2 × 10 ml) to remove acids and phenols. (The emulsions observed with $CHCl_3$ in the original procedure were not encountered here.) The EtOAc was removed by evapn and the isolation continued as previously described except for the following changes: (i) The pH was adjusted to 10–11 instead of 9–10. (ii) The EtOAc solution of the residue from the Et_2O extract was washed with fresh $NaHCO_3$ solution, pH *ca* 8.5. (After standing one day it rose to *ca* 9.5.) The solution was concd to dryness in a sublimation tube which was heated over the range 130–180° at 0.1 Torr for 2–3 hr. The sublimate, almost entirely crystalline, was recrystallized from EtOAc–hexane to give isofraxidin, mp 145–147°. As before, final purification was by *O*-methylation to yield TMC.

In the 1985 experiments recorded in Table 2 a blank experiment was performed in parallel, in which a measured amount of labelled isofraxidin, in EtOAc solution, was added to the plant material before its work-up. The mixture was then submitted to the isolation procedure as modified above for the recovery of any isofraxidin derived from hydrolysis of puberulin.

Mass spectrometry. Electron impact mass spectra were obtained with a Vacuum Generators V916-F single focussing mass

spectrometer, and operating desorption probe in the electron impact mode. Methane chemical ionization spectra (CH_4 Cl) were obtained with a Finnigan 4500 mass spectrometer, and gas chromatographic separation was effected with a Pierce OV-45 packed column (Box 117, Rockford, IL 61105, U.S.A.). The acidic fraction of *A. puberula* analysed by these techniques was that obtained by extraction of the chloroform solution of the original procedure with 4% NaOH [3].

Degradation of TMC The methyl carbons of TMC were recovered, after demethylation with HI, as Et_3MeNI by the previously published procedure [3].

Measurement of carbon-14 All samples were analysed for ^{14}C by scintillation spectrometry. Et_3MeNI was counted in Bray's solution in the presence of $Na_2S_2O_3$ [3], and all other samples in a toluene-based medium.

Acknowledgements—The studies here were supported by grants from the Natural Sciences and Engineering Research Council of Canada (A2487) and the South African Council for Scientific and Industrial Research. We are very grateful to Dr W. E. Campbell of the University of Cape Town for a generous sample of capensin, Dr Glenys Foster, formerly of the Ontario Ministry of the Environment, for providing the CI mass spectra and Dr Ben Khouw of York University for the electron impact mass spectrum.

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