

Biosynthesis of Daphnetin in *Daphne mezereum* L.*

Stewart A. Brown

Department of Chemistry, Trent University, Peterborough, Ont., Canada K9J 7B8

Z. Naturforsch. **41c**, 247–252 (1986); received November 4, 1985

Biosynthesis, Coumarins, *Daphne mezereum*, Daphnetin

Shoots of *Daphne mezereum* synthesized daphnetin (7,8-dihydroxycoumarin) more efficiently from [2-¹⁴C]umbelliferone (7-hydroxycoumarin) than from [2-¹⁴C]*p*-coumaric acid, and [2-¹⁴C]caffeic acid was more poorly utilized still. These findings do not support the idea of derivation of daphnetin via hydroxylation of the caffeic acid ring at the 2 position, followed by lactone ring formation; instead they are consistent with the concept of daphnetin formation by an additional hydroxylation of umbelliferone at C-8. Umbelliferone was recovered with little ¹⁴C dilution from emulsin-hydrolysed extracts of shoots fed labelled umbelliferone, and TLC of extracts from untreated shoots revealed two substances yielding umbelliferone on hydrolysis. Evidence is presented from TLC and HPLC analysis that one of these is skimmin (7-O-β-D-glucosylumbelliferone), not previously reported from *Daphne*. The tracer experiments further support the theory that umbelliferone is the general precursor of coumarins bearing two or more hydroxyl functions on the aromatic ring.

Introduction

Approximately 70 coumarins with the 7,8 oxygenation pattern⁺ have been reported to be naturally occurring [1], over half being furanocoumarins derived from xanthotoxol. Little attention has been devoted to the biosynthesis of these coumarins, apart from xanthotoxin, whose origin is well understood [1]. Of the simple, 7,8-oxygenated coumarins only hydrangetin (**3**, Scheme 2) appears to have been studied. It does not arise, at least in *Hydrangea macrophylla* [2], through introduction of a hydroxyl group in the 2' position of ferulic acid (**1a**) (Scheme 1B), analogously to the *ortho* hydroxylation in the 6' position (Scheme 1A) which yields scopoletin in tobacco [1]. Instead, there is evidence that in *H. macrophylla*, umbelliferone (**5a**, Scheme 2), presumably formed via *p*-coumaric acid (**4**), is an intermediate in hydrangetin biosynthesis [2–4]. If this is the case, the implication is that daphnetin (**6a**, Scheme 2) is synthesized by 8-hydroxylation of umbelliferone, with 8-O-methylation the ultimate reaction of the biosynthetic sequence (Scheme 2). The origin of daphnetin has not been examined.

The present report describes an investigation of the formation of daphnetin in *Daphne mezereum* L.

Abbreviations: DMC, 7,8-dimethoxycoumarin; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

* The studies reported here were supported by an operating grant from the Natural Sciences and Engineering Research Council of Canada, No. A2487.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/0300–0247 \$ 01.30/0

(Thymelaeaceae), a hardy perennial shrub, where it occurs together with its 7-β-D-glucoside, daphnin (**6b**) and daphnetin glucoside (**6c**), the 8-O-β-D-glucosylated isomer [1].

In addition to examining the probable role of umbelliferone as an intermediate in daphnetin biosynthesis, I have investigated the possibility of the involvement of caffeic acid (**1b**), in a manner analogous to the reactions of the hypothetical Scheme 1B.

Experimental

Preparation of labelled substrates

Syntheses of the [2-¹⁴C]hydroxycinnamic acids [5] and [2-¹⁴C]umbelliferone [6] have been described in earlier publications.

Cultivation of the plant and labelled substrate feedings

A single *D. mezereum* shrub, purchased commercially and grown in a garden, was used for all experiments. Shoots were harvested during the period when it had reached a height of approx. 60–100 cm.

Radioactive substrates were administered twice in early August and once in early June. Shoots were cut from the plant and the cut ends trimmed under water just before feeding. The hydroxycinnamic acids were dissolved with the aid of minimal sodium bicarbonate and the umbelliferone with 0.5% potassium hydroxide, the final volume in all cases approximating 1 ml. Feedings were done in a greenhouse in warm weather and bright sunlight, and in all cases absorption

+ For numbering see structures in Scheme 1.



Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung“) beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition “no derivative works”). This is to allow reuse in the area of future scientific usage.

through the cut ends of the shoots was complete in 30–45 min. After absorption of a rinse of distilled water the shoots were placed in Hoagland's plant nutrient solution and allowed to metabolize 24–27 h without artificial light.

Isolation of daphnetin

The early stages of the isolation were similar to those described by Satō and Hasegawa [7] for *D. odora*. The material was first heated 0.5 h with refluxing 25% ethanol and then comminuted in a blender. In the June feeding the stems as well as leaves were extracted, but in the August experiments, as the stems had become highly lignified and difficult to comminute, only the leaves were used. The residues after filtration were further extracted with boiling water. The combined extracts were evaporated to dryness in vacuo and the residues after this step were extracted 0.5 h with refluxing absolute ethanol. The undissolved material in each case was then taken up in water and the resulting solutions extracted with ethyl acetate. The extracts, which should have contained any free coumarins, were not examined.

The aqueous residue from each experiment was treated for several hours with approx. 1 mg of β -glucosidase (commercial emulsin, Sigma Chemical Co., St. Louis, U.S.A.) to hydrolyse glucosides of daphnetin, and the hydrolysate was exhaustively extracted with ether in a continuous extractor. In the initial (August, 1984) experiment an attempt was made at preliminary purification of the daphnetin from the *p*-coumaric acid feeding, before derivatization, on a silica column, but as the process was slow and the recovery low, this step was abandoned and the aglycones from the concurrent umbelliferone feeding were methylated to DMC with methyl iodide in the manner previously described for isofraxidin [5], without preliminary purification. The product was sublimed at 110 °C, < 1 Torr and then chromatographed on a silica chromatoplate, 20 × 20 cm, 1 mm thick, developed twice with hexane-ethyl acetate, 3:1. A prominent faster-moving band of herniarin, the product of umbelliferone O-methylation, moved well clear of the DMC band, and there were several minor bands. The two major bands were scraped from the plate and extracted into hot acetone. The daphnetin recovered from the column after the *p*-coumaric acid feeding was similarly

purified. The residues from removal of the acetone were resublimed at 100 °C, < 1 Torr, to give colourless crystals, m.p. 116.5–118 °C (cor.); lit. m.p. 114–116 °C [1]. Because of the low recoveries the DMC from the *p*-coumaric acid feeding was not further purified, and the quantity was determined from a standard curve by UV absorption at 318 nm, as were those of the herniarin bands, at 325 nm. The DMC from the umbelliferone feeding was weighed, carrier was added, and the material recrystallized to constant specific activity from hexane-ethyl acetate.

In both of the 1985 feeding experiments a modified purification procedure was adopted for the aglycones from the emulsin hydrolysis. A portion of this material, predominantly daphnetin, was spread in a band on a silica gel G (Macherey-Nagel) chromatoplate, and a preliminary purification of the daphnetin was conducted by three or four successive developments in chloroform-methanol, 49:1. Umbelliferone moved ahead of daphnetin and most of it could be separated from the dihydroxycoumarin. The daphnetin and umbelliferone bands were excised and the coumarins recovered by hot acetone extraction. The daphnetin samples were methylated to DMC, which was sublimed as before; the umbelliferone was sublimed as such, at 210 °C, < 1 Torr. Final purification was by reversed phase HPLC [8]. DMC was eluted with 25% aqueous acetonitrile, and detected by UV absorption at 360 nm. In this system herniarin (7-methoxycoumarin) trails and is cleanly separated from DMC; thus there is no possibility of the latter's contamination by tailing of herniarin formed in the methylation step from traces of umbelliferone carried over from the silica chromatoplate. (Removal of all traces of umbelliferone from the daphnetin is especially critical in the [2-¹⁴C]umbelliferone feeding experiment, owing to the ease with which administered labelled umbelliferone can be glucosylated to skimmin (**5b**) in plant cells [9], with the potential of regenerating high-specific-activity aglycone upon emulsin hydrolysis.) Examined under UV, a normal-phase silica chromatoplate of a sample of the dimethoxycoumarin band from the HPLC column, developed in iso-octane-ethyl acetate, 3:1, showed no extraneous fluorescent or quenching spots. Umbelliferone was eluted with water-methanol-acetic acid, 80:20:1, and detection was at 350 nm. Again, no extraneous fluorescent or quenching spots were detected by TLC of the recovered umbelliferone on silica with chloroform-acetone, 9:1.

Analysis of ^{14}C

The purified coumarins, dissolved in methanol, were analysed for ^{14}C by scintillation spectrometry in a toluene-based system.

Examination of *D. mezereum* extract for umbelliferone glycosides

D. mezereum leaves (12.5 g fresh wt.) were collected in the late summer and treated as described above to yield an ethyl acetate-extracted aqueous solution. After filtration a sample of this solution was spread in a band on a silica gel G chromatoplate, 20 × 20 cm × 1 mm thick, and the chromatogram developed in water-saturated 1-butanol with a reference marker of authentic skimmin. The developed chromatogram was arbitrarily sectioned into eight bands, most of them corresponding to fluorescent substances, which were then extracted at room temperature with 25% aqueous ethanol. After concentration of each extract to ~ 1 ml, the aqueous residue was treated overnight with emulsin, and two samples were examined by TLC on silica gel G, developed in chloroform–acetone, 9:1, and in toluene–ethyl acetate–acetic acid, 5:4:1. As a band of R_f 0.5, corresponding in mobility to authentic skimmin, did not exhibit the characteristic purple fluorescence of this glucoside, giving a more bluish shade, it appeared that another fluorescent compound with the same R_f value could have been masking this fluorescence, and further separation was therefore attempted by

another procedure. The extract of this band was further concentrated and submitted to reversed-phase HPLC on a Waters Nova-Pak C_{18} column as previously described [8], with 5% aqueous acetonitrile as the eluent.

Results and Discussion

The seasonal dynamics of the accumulation and distribution of hydroxycoumarins in *D. mezereum* have been extensively studied by Koshleva and Nikonov [10, 11] in plants growing near Moscow. In addition to free umbelliferone and daphnetin, these workers identified the glucoside daphnin (**6b**) and the dicoumarin, daphnoretin. Daphnin was the main component and existed in relatively high concentration in the leaves, flowers, and branch bark. The maximal polyphenol content was observed during the period of leaf formation and at the end of the growth period, presumably in mid-spring and late summer, respectively.

Radiotracer feeding experiments

On the assumption that the Soviet observations are also valid in the geographic location of the present experiments, and that they reflect more rapid synthesis at these times, feedings of labelled substrates were done as near as convenient to these two periods of the year.

Table I presents the results of three experiments in which ^{14}C -labelled substrates were administered to

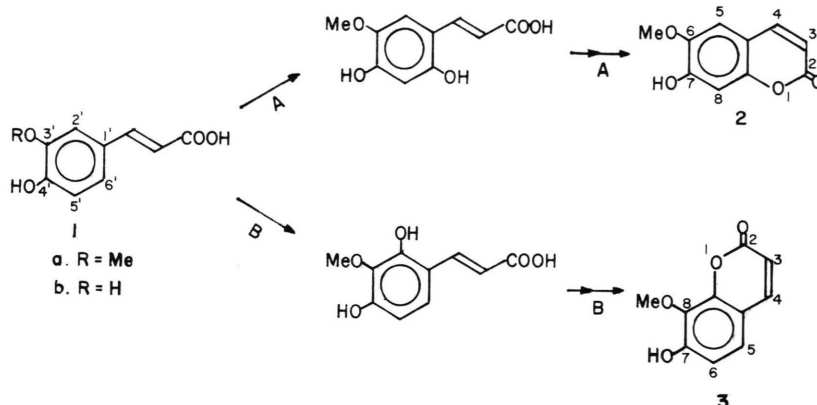
Table I. Comparison of umbelliferone, *p*-coumaric acid, and caffeic acid as precursors of coumarin aglycones in *Daphne mezereum*.

Date of feeding	Substrate administered ^a	Name	Aglycone recovered [Bq/mmol]	Dilution of ^{14}C
1984 08	[2- ^{14}C]umbelliferone	daphnetin ^b	15 500	381
		umbelliferone ^c	6.08×10^6	0.97
1985 06	[2- ^{14}C] <i>p</i> -coumaric acid	daphnetin ^b	3 990	1 470
	[2- ^{14}C]umbelliferone	daphnetin ^b	24 200	244
		umbelliferone	5.73×10^5	10.3
	[2- ^{14}C] <i>p</i> -coumaric acid	daphnetin ^b	15 100	392
1985 08	[2- ^{14}C]umbelliferone	umbelliferone	53 100	111
		daphnetin ^b	11 800	501
		umbelliferone	2.97×10^6	2.0
	[2- ^{14}C] <i>p</i> -coumaric acid	daphnetin ^b	617	9 580
		umbelliferone	27 500	215
	[2- ^{14}C]caffeic acid	daphnetin ^b	512	11 500
	[2- ^{14}C]caffeic acid	daphnetin ^b	213	27 700

^a 5.90 ± 0.04 MBq/mmol in all cases.

^b Counted as DMC.

^c Counted as herniarin.



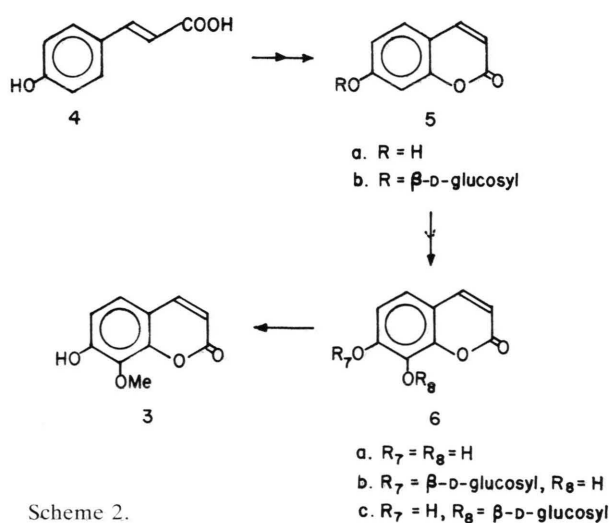
Scheme 1.

D. mezereum and compared as precursors of the aglycone of daphnetin glucosides. The dilution of the label in *p*-coumaric acid (**4**) whose hydroxylation at C-2' is known to be the committed step in the biosynthesis of 7-hydroxycoumarins [1], has been taken as the standard for comparison of precursor efficiency. In the experiment including duplicate feedings of [2-¹⁴C]caffeic acid the carbon-14 dilutions for this substrate were, on average, over twice as great as that for *p*-coumaric acid, giving no support to the idea of a route analogous to Scheme 1B, and in accord with the findings of Kindl and Billek [2] for hydrangetin.

Umbelliferone, in contrast, was consistently better incorporated into daphnetin than was *p*-coumaric acid. This was especially marked in the 1985 08 (Au-

gust) experiment, where the difference was a factor of 19; in the 1984 08 experiment it was about four, whereas in the 1985 06 (June) feeding it was less than a factor of two. This difference, which one might suspect *prima facie* to be a seasonal variation, is not readily explicable on the basis of available data. It could be supposed that the level of *p*-coumaric acid 2'-hydroxylase decreased in late summer relative to that of the presumed umbelliferone 8-hydroxylase yielding daphnetin. But this should also have been expected to result in a greatly decreased incorporation of label from *p*-coumaric acid into umbelliferone which was, in fact, not nearly so marked. Alternatively one might postulate a greatly reduced size of the umbelliferone pool in June, substantially reducing the dilution of label in *p*-coumaric acid during its passage through this pool. Reference to the data of Table I reveals, however, that in the August feedings of [2-¹⁴C]umbelliferone the dilution values for the recovered umbelliferone were in the range of one (*i.e.*, no dilution) to two, indicating that the combined pools of free umbelliferone and its glycosides were very small, whereas in the June feeding the value was ten, pointing to a *greater* pool size at this season.

Despite this difficulty of interpretation, the experimental results reported here are quite consistent with the biosynthetic route: **4** → **5a** → **6a** of Scheme 2, and contraindicate introduction of the C-8 hydroxyl group of daphnetin before elaboration of the coumarin nucleus. As umbelliferone occurs in *D. mezereum* [1], the demonstration of its precursor role in daphnetin formation implies that it is a natural intermediate in this reaction sequence. Daphnetin thus joins a growing list of coumarins oxygenated at two

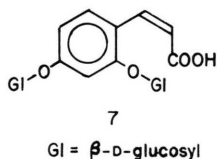


Scheme 2.

or more positions of the benzene ring for which there is strong evidence of derivation via umbelliferone, and it is becoming increasingly probable that their formation from umbelliferone is a general phenomenon.

The nature of bound umbelliferone in D. mezereum

The recovery of umbelliferone following emulsin treatment of the plant extract clearly suggests, as implied above, the existence in *D. mezereum* of a glycosidic form or forms of this coumarin. The most common glycoside yielding umbelliferone is skimmin (**5b**), which occurs in species of several plant families but apparently has not been reported from any species of *Daphne* [1]. *H. macrophylla* contains *cis*-2,4- β -D-glucosyloxycinnamic acid (**7**), whose aglycone spontaneously lactonizes to umbelliferone [12], as well as neohydrangin, a substance yielding umbelliferone on hydrolysis but whose structure has not been elucidated [13]. Information as to the nature of the bound umbelliferone in *D. mezereum* was sought through examination of unhydrolysed 25% ethanol extract from which free coumarins had been removed.



A portion of this solution, after TLC in water-saturated butanol, gave numerous fluorescent bands when observed under UV. One broad band centred at about R_f 0.4, after extraction and hydrolysis with emulsin, yielded an aglycone which corresponded in R_f value and fluorescence to authentic daphnetin when examined by TLC on silica in two solvent systems. This band presumably contained daphnin, of which no reference standard was available. In addition, the hydrolysates from the bands moving at about R_f 0.5 and 0.1 in this solvent system both gave material after hydrolysis which was indistinguishable from authentic umbelliferone by its R_f and fluorescence after TLC in two solvent systems. The unhydrolysed material moving at R_f 0.5 in aqueous butanol had the same mobility as authentic skimmin. This band was further examined by reversed-phase HPLC [8]. A small peak emerged at a retention vol-

ume of 10.5 ml, identical to that of skimmin and, upon collection, TLC in aqueous butanol confirmed an identical R_f value to that of skimmin. The smaller umbelliferone-yielding TLC band of R_f 0.1 was not further examined.

Although concentrations were too low for more rigorous identification procedures with the amount of extract available, the above chromatographic data are regarded as strong evidence for the existence of skimmin in the leaves of *D. mezereum*, and there are indications as well of a second bound form of umbelliferone, of unknown structure.

Possible biosynthetic significance of bound umbelliferone

The present studies were not designed to examine the question of when the glucoside linkage at the 7-hydroxyl of daphnin is formed. Nevertheless, if it is at the *p*-coumaric stage, and specificity of the enzyme system metabolizing the glycosidic product is assumed, then administered free umbelliferone should have been, at best, less effectively utilized as a daphnin precursor than was *p*-coumaric acid. The present observations rule this out. They are, however, consistent with 7-O-glucosylation of either umbelliferone or daphnetin, represented, respectively, by the sequences **4** \rightarrow **5a** \rightarrow **5b** \rightarrow **6b** and **4** \rightarrow **5a** \rightarrow **6a** \rightarrow **6b** of Scheme 2. In an earlier investigation [9] of the formation of the aesculetin (6,7-dihydroxycoumarin) glucoside, cichoriin, in which the sugar is linked via the 7-hydroxyl, as in daphnin, it was suggested that umbelliferone could be glucosylated to skimmin in a rate-limiting reaction, with the skimmin being metabolized to another product, possibly cichoriin, in a faster reaction. Failure of skimmin to accumulate in appreciable amounts in *D. mezereum* leaves is consistent with an analogous reaction sequence leading to daphnin, but here, also, it would appear that enzyme-level experiments will be necessary to decide between this route and the alternative in which daphnetin is an intermediate.

Acknowledgement

I wish to thank Dr. Warren Steck of the Plant Biotechnology Institute, Saskatoon, for a gift of skimmin.

- [1] R. D. H. Murray, J. Méndez, and S. A. Brown, *The Natural Coumarins: Occurrence, Chemistry, and Biochemistry*, John Wiley & Sons Ltd., Chichester, U.K. 1982.
- [2] H. Kindl and G. Billek, *Monatsh. Chem.* **95**, 1044 (1964).
- [3] B. Bohm, R. K. Ibrahim, and G. H. N. Towers, *Can. J. Biochem.* **39**, 1389 (1961).
- [4] S. A. Brown, G. H. N. Towers, and D. Chen, *Phytochemistry* **3**, 469 (1964).
- [5] S. A. Brown, D. E. A. Rivett, and H. J. Thompson, *Z. Naturforsch.* **39c**, 31 (1984).
- [6] S. A. Brown, *Phytochemistry* **2**, 137 (1963).
- [7] M. Satô and M. Hasegawa, *Phytochemistry* **11**, 657 (1972).
- [8] H. J. Thompson and S. A. Brown, *J. Chromatogr.* **314**, 323 (1984).
- [9] S. A. Brown, *Can. J. Biochem. Cell Biol.* **63**, 292 (1985).
- [10] L. I. Koshleva and G. K. Nikonov, *Tr. Vses. S'ezda Farm., 1st* 1967 (Pub. 1970) 233; *Chem. Abstr.* **75**, 126577 (1971).
- [11] L. I. Koshleva and G. K. Nikonov, *Farmatsiya (Moscow)* **19**, 36 (1970); *Chem. Abstr.* **74**, 10324 (1971).
- [12] D. J. Austin and M. B. Meyers, *Phytochemistry* **4**, 255 (1965).
- [13] V. Plouvier, *C.R. Hebd. Seances Acad. Sci., Ser. C* **252**, 312 (1961); *Chem. Abstr.* **55**, 12393i (1961).