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FURTHER STUDIES ON THE BIOSYNTHESIS OF MANGIFERIN IN *ANEMARRHENA ASPHODELOIDES*: HYDROXYLATION OF THE SHIKIMATE-DERIVED RING*

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Key Word Index—*Anemarrhena asphodeloides*; Liliaceae; biosynthesis; hydroxylation; *C*-glucosylxanthones; mangiferin; isomangiferin; iriflophenone; maclurin.

Abstract—The hydroxylation at C-3′ of maclurin, an intermediate in mangiferin biosynthesis, has been studied. Labelled cinnamic acid, *p*-coumaric acid, caffeic acid, iriflophenone and maclurin were fed to *Anemarrhena asphodeloides*. Cinnamic acid and *p*-coumaric acid were better precursors than caffeic acid for mangiferin, and iriflophenone as well as maclurin was effectively incorporated into mangiferin and isomangiferin. These results show that maclurin is biosynthesized via hydroxylation of iriflophenone derived from *p*-coumarate in this plant.

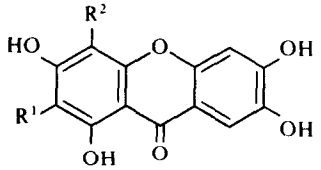
INTRODUCTION

In previous reports [1–3], we proposed that the *C*-glucosylxanthones mangiferin (**1**) and isomangiferin (**2**) in *Anemarrhena asphodeloides* (Liliaceae) were biosynthesized via maclurin (**3**) derived from *p*-coumarate and malonate, and that *C*-glucosylation to **1** and **2** occurs at the stage of **3** prior to the formation of the xanthone nucleus. This paper deals with the hydroxylation at C-3′ in maclurin (**3**), an intermediate in mangiferin biosynthesis.

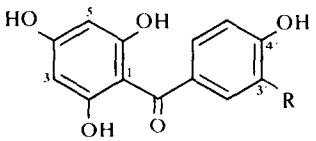
RESULTS AND DISCUSSION

Labelled compounds were fed to the aerial parts of *A. asphodeloides*. At first, parallel feeding experiments with labelled cinnamic acid, *p*-coumaric acid and caffeic acid, and competitive feeding experiments with a mixture of *p*-[ring-3,5-^{3}H]coumaric acid and [2-^{14}C]caffeic acid were performed (Tables 1 and 2). Comparison of incorporation ratio of labelled compounds into mangiferin (**1**) and isomangiferin (**2**) indicates that cinnamic acid and *p*-coumaric acid were better precursors than caffeic acid for **1** and **2**, and that the hydroxylation at C-3′ in maclurin (**3**) occurs at the late stage in biosynthesis. Thus [1,3,5-^{14}C]iriflophenone (**4**) as a possible precursor of **3** was fed simultaneously to the plants in parallel with [1,3,5-^{14}C]maclurin (**3**). Labelled **4** as well as **3** was effectively incorporated into **1** and **2**, and the radioactivity in **1** was largely present in the aglycone moiety. These results show that maclurin (**3**) was biosynthesized via C-3′ hydroxylation of iriflophenone (**4**) derived from *p*-coumarate and malonate. Iriflophenone (**4**) co-occurs with **3** in the branches of *Morus alba* [4] and also with **1**, **2** and irisxanthone (1,3,6-trihydroxy-5-methoxyxanthone-2-*C*-glucoside) in the rhizomes of *Iris florentina* ([5, 6]; Fujita, M. and Inoue, T., unpublished results). Recently the 4-methyl ether of **4** [7] was isolated from the rhizomes of *A. asphodeloides* together with **1** and **2**. These findings support our tracer work.

It has been demonstrated that flavonoids possessing 3′,4′-dihydroxyl groups in the B-ring [8] and hispidin (4-hydroxy-6-(3,4-dihydroxystyryl)-2-pyrone) [9, 10] could be biosynthesized from both *p*-coumarate and caffeate. In a previous paper [2], we reported that the biosynthesis of mangiferin (**1**) seems to be closely related to that of *C*-glucosylflavones. The aglycone (**5**) of **1** is probably formed by a pathway similar to hispidin biosynthesis. Considering the appreciable incorporation ratio of [2-^{14}C]- and [3-^{14}C]caffeic acid into **1** and **2**, maclurin also might be biosynthesized from caffeate. In order to obtain evidence for the participation of caffeic acid in mangiferin



1 R^1 = Glc, R^2 = H
2 R^1 = H, R^2 = Glc



3 R = OH
4 R = H

*Part 3 in the series "Biosynthesis of Mangiferin in *Anemarrhena asphodeloides*". For Part 2 see ref. [3].

Table 1. Incorporation of labelled precursors into mangiferin and isomangiferin in *A. asphodeloides*

Experiment	Precursor	Amount fed (μCi)	Amount fed (mg)	*C*-Glucosylxanthones Incorp. (%)	Dilution	Sp. act. (dpm/mM)	Degradation products Sp. act. (dpm/mM)	Ratio* (%)
1.	[2-^{14}C]Cinnamic acid	9.7	10	M 2.20	780	4.07×10^5	AG 4.03×10^5	99.0
				IM 0.24	1200	2.66×10^5	AG 2.59×10^5	97.4
2.	[3-^{14}C]Cinnamic acid	8.5	10	M 2.25	740	3.74×10^5		
3.	*p*-[2-^{14}C]Coumaric acid	10.9	10	M 1.98	930	4.25×10^5		
4.	[2-^{14}C]Caffeic acid	27.8	10	M 0.39	4900	2.27×10^5	AG 2.16×10^5	95.2
							P 2.09×10^5	92.1
5.	[3-^{14}C]Caffeic acid	32.0	10	M 0.43	4800	2.68×10^5		
				IM 0.03	8100	1.58×10^5		
6.	[1,3,5-^{14}C]Iriflophenone	0.76	5	M 0.52	3400	2.43×10^4	AG 2.33×10^4	95.9
				IM 0.06	4300	1.92×10^4		
7.	[1,3,5-^{14}C]Maclurin	0.26	5	M 0.61	3900	7.82×10^3	AG 7.56×10^3	96.7
				IM 0.05	5700	5.31×10^3		

Experiments 1–5 and 6 + 7 are parallel experiments. Feeding period: 50 hr. M: Mangiferin; IM: isomangiferin; AG: aglycone; P: phloroglucinol.

*Ratios show % to the specific activity of *C*-glucosylxanthones.

Table 2. Ratio of ^3H and ^{14}C activities in the precursor and mangiferin after feeding a mixture of *p*-[ring-3,5-^3H] coumaric acid and [2-^{14}C]caffeic acid

Experiment*	$^3H/^{14}C$†	Incorp. (%)	Dilution	Sp. act. (dpm/mM)	$^3H/^{14}C$‡
8-(i)	2.49	^3H: 0.50	7100	9.26×10^4	16.7
		^{14}C: 0.078	25000	1.11×10^4	
8-(ii)	2.60	^3H: 1.38	2700	2.42×10^5	16.2
		^{14}C: 0.22	9300	2.98×10^4	

*Feeding period: (i) 15 hr, (ii) 50 hr.

†Ratio in the precursor.

‡This ratio is corrected for the loss of half of ^3H on arylhydroxylation during biosynthesis.

biosynthesis, the aglycone of **1** obtained after feeding [2-^{14}C]caffeic acid was degraded to phloroglucinol. If [2-^{14}C]caffeic acid were utilized for the formation of the aglycone (**5**) without randomization, the label would be introduced into the phloroglucinol ring of **5**. As shown in Table 1, the radioactivity was almost exclusively present in the phloroglucinol moiety of **5**. The results suggest the occurrence of a subroute by which mangiferin (**1**) and isomangiferin (**2**) could be biosynthesized directly from caffeic acid.

EXPERIMENTAL

Preparation of radioactive compounds. The syntheses of *p*-[2-^{13}C]coumaric acid [11], *p*-[ring-3,5-^3H]coumaric acid [2], [2-^{14}C]caffeic acid [11] and [1,3,5-^{14}C]maclurin (**3**) [3] have been described previously. [3-^{14}C]Cinnamic acid (2.78×10^8 dpm/mM) was prepared by the dilution of [3-^{14}C]cinnamic acid (1.03×10^{11} dpm/mM, Commissariat A L'Energie Atomique). [2-^{14}C]Cinnamic acid (3.18×10^8 dpm/mM) was synthesized from [2-^{14}C]malonic acid (50 μCi, 3.77×10^{10} dpm/mM, Radiochemical Centre, Amersham), malonic acid (50 mg) and benzaldehyde (40 mg) by the conventional method [12]. [3-^{14}C]Caffeic acid (1.28×10^9 dpm/mM) was obtained by the condensation of malonic acid (40 mg) and [carbonyl-^{14}C]protocatechualdehyde (1.22×10^9 dpm/mM, 35 mg) which was synthesized from K^{14}CN in accordance with the method of ref. [13]. [1,3,5-^{14}C]Iriflophenone (**4**) was prepared as follows [14]. Na-dried Et_2O (3 ml) was added to a mixture of [2,4,6-^{14}C]phloroglucinol [3] (120 mg), *p*-hydroxybenzonitrile (120 mg) and fused $ZnCl_2$ (1 g), and the mixture was saturated with dry HCl for 4 hr. After standing overnight, the reaction mixture was diluted with dil. H_2SO_4 and washed with Et_2O. The aq. layer was gently warmed to remove Et_2O, and allowed to stand overnight. The resulting yellow-brown precipitate was collected and refluxed with H_2O for 2 hr. After cooling, the precipitate was filtered off, and the filtrate extracted with EtOAc. The extract was chromatographed on Si gel (10 g) using $CHCl_3$–MeOH (10:1) as eluant, and the fractions containing **4** were combined and recrystallized from H_2O to give [1,3,5-^{14}C]iriflophenone (**4**) (14.8 mg, 8.34×10^7 dpm/mM). The purity of the radioactive compounds was examined by radiochromatogram (Aloka JTC-201) on TLC. The radioactivity was measured with an Aloka LSC-602 liquid scintillation counter, in a POP–POPOP–naphthalene–dioxane scintillator solution.

Feeding experiments. Labelled cinnamic acid and its derivatives were dissolved in a minimum amount of 0.1 N $NaHCO_3$, and benzophenone derivatives in a minimum amount of 0.1 N NaOH, and then the precursor solution was administered to the aerial parts of *A. asphodeloides* essentially as described previously [2]. After feeding for 15 and 50 hr, the plants were cut into small pieces and dried at 60°. Isolation of **1** and **2** from plant material, and their degradation with HI to the aglycone (**5**), as well as that of **5** by alkali fusion to phloroglucinol, were performed according to reported procedures [3].

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