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Biosynthesis of Mangiferin in *Anemarrhena asphodeloides* Bunge. I. The Origin of the Xanthone Nucleus[1]

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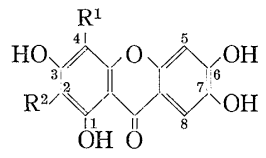
Phenylalanine-1-^{14}C, -2-^{14}C and -3-^{14}C, and malonic acid-2-^{14}C were efficiently incorporated into mangiferin (**1**) in *Anemarrhena asphodeloides*. In the case of feeding with phenylalanine-1-^{14}C, -2-^{14}C and malonic acid-2-^{14}C, the radioactivity of **1** was localized in the phloroglucinol ring. Furthermore, cinnamic acid-3-^{14}C and *p*-coumaric acid-2-^{14}C were also incorporated into **1** and isomangiferin (**2**), but benzoic acid-, *p*-hydroxybenzoic acid- and protocatechuic acid-(carboxyl-^{14}C) were essentially not incorporated into **1** or **2**. In addition to the above data, doubly labelled *p*-coumaric acid was incorporated into **1** without change of the T/^{14}C ratio.

These results show that the aglycone of **1** and **2** was biosynthesized from *p*-coumarate (C_6—C_3) and two malonates (C_4).

Keywords——*Anemarrhena asphodeloides* Bunge; biosynthesis of xanthones; mangiferin; isomangiferin; *p*-coumaric acid-2-^{14}C and -(ring-3,5-T_2); *p*-hydroxybenzoic acid-(carboxyl-^{14}C) and -3,5-T_2; protocatechuic acid-(carboxyl-^{14}C); benzoic acid-(carboxyl-^{14}C); phenylalanine-1-^{14}C, -2-^{14}C and -3-^{14}C; malonic acid-2-^{14}C

Recently a large number of xanthones has been found in higher plants and as fungal metabolites.[3]

It has been shown by the isotope-tracer method that naturally occurring xanthones are biosynthesized *via* an intermediate with a benzophenone nucleus derived wholly from polyketide in fungi,[4] and from shikimate-polyketide in higher plants.[5,6] The biosynthesis of xanthones in higher plants has been studied in *Gentiana lutea*. Fross and Rettig[5] demonstrated that the phloroglucinol ring of gentisin (gentisein 7-methyl ether) is derived from acetate, and latter, Gupta and Lewis[6] reported that gentisein (1,3,7-trihydroxyxanthone) and related xanthones are biosynthesized by oxidative coupling of a benzophenone derived from phenylalanine by the loss of two carbon fragments.



1: R^1=H, R^2=glucosyl
2: R^1=glucosyl, R^2=H

Chart 1

Although the known distribution of xanthones and their *O*-glycosides in plants is limited so far to several families, a xanthone *C*-glucoside, mangiferin (**1**) is more widely distributed in the plant kingdom.[3a,7] Mangiferin (**1**) and related *C*-glucosylxanthones have 5,6- or 6,7-dihydroxy groups, which are expected oxidation patterns in aromatic rings derived from shikimate, but many other xanthones, including "*Gentiana* xanthones," have different oxidation patterns.[3a] Therefore, the xanthone nucleus

1) A part of this study has been reported as a preliminary communication: M. Fujita and T. Inoue, *Tetrahedron Lett.*, **1977**, 4503.
2) Location: *Ebara 2-4-41, Shinagawa-ku, Tokyo, 142, Japan.*
3) *a*) I. Carpenter, H.D. Locksley, and F. Scheinmann, *Phytochemistry*, **8**, 2013 (1969); K. Hostettman and H. Wagner, *ibid.*, **16**, 821 (1977); *b*) T.K. Devon and A.I. Scott, "Handbook of Naturally Occurring Compounds," Vol. I, by Academic Press Inc., New York, 1975, p. 293.
4) B. Frank, F. Hüper, and D. Gröger, *Chem. Ber.*, **101**, 1970 (1968); A.J. Birch, J. Baldas, J.R. Hlubucek, T.J. Simpson, and P.W. Westerman, *J. Chem. Soc. Perkin I*, **1976**, 898, and references cited therein.
5) H.G. Fross and A. Rettig, *Z. Naturforsch.*, **19b**, 1103 (1964).
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7) C.A. Williams, *Phytochemistry*, **18**, 803 (1979).

of mangiferin (**1**) and related *C*-glucosylxanthones might be biosynthesized *via* a route different from that of "*Gentiana* xanthones."

We have studied the biosynthesis of **1** and its position isomer, isomangiferin (**2**), in *Anemarrhena asphodeloides*, and propose that the xanthone nucleus of these *C*-glucosides is biosynthesized from *p*-coumarate and two malonates.

Results and Discussion

Anemarrhena asphodeloides Bunge (Liliaceae) was used as the plant material; its aerial parts and rhizomes are known to contain mangiferin (1,3,6,7-tetrahydroxyxanthone 2-*C*-glucoside) (**1**) and isomangiferin (1,3,6,7-tetrahydroxyxanthone 4-*C*-glucoside) (**2**).[8] The labelled compounds were prepared as follows. *p*-Hydroxybenzoic acid-3,5-T_2 was obtained by the oxidation of *p*-hydroxybenzaldehyde-3,5-T_2[9] with silver nitrate in alkali solution.[10] Protocatechuic acid-(carboxyl-^{14}C) was obtained by the demethylation of anisic acid-(carboxyl-^{14}C). *p*-Coumaric acid-(ring-3,5-T_2) was synthesized from *p*-hydroxybenzaldehyde-3,5-T_2 and malonic acid.[11] *p*-Coumaric acid-2-^{14}C was prepared by the method described previously.[12]

Various labelled compounds (shown in Tables I—III) were fed to the excised aerial parts of the plants. After feeding for 15 and 70 hr, the radioactive mangiferin (**1**) was isolated from the plant materials, and in many cases degraded with hydroiodic acid to the aglycone (1,3,6,7-tetrahydroxyxanthone) (**3**), followed by acetylation and purification as the tetraacetate. In some experiments, radioactive isomangiferin (**2**) was also isolated from the plant materials.

Table I. Incorporation of Phenylalanine-1-^{14}C, -2-^{14}C, -3-^{14}C and Malonic Acid-2-^{14}C into Mangiferin and Radioactivity of the Degradation Products

Expt.	Precursors		Amount fed (μCi)	Amount fed ($\times 10^{-2}$ mg)	Yield (mg)	Incorporation (%)	Sp. act. (dpm/mM) Mangiferin	Aglycone[a] (tetraacetate)	Phloroglucinol[a]
	Phenylalanine								
1.	-1-^{14}C	(a)	12.5	3.5	490	1.15	2.74×10^5	2.67×10^5(97.4)	2.65×10^5(96.7)
		(b)	25	7	295	2.98	2.37×10^6	2.31×10^6(97.5)	
2.	-2-^{14}C	(a)	12.5	8	503	1.22	2.84×10^5	2.81×10^5(98.9)	2.77×10^5(97.5)
		(b)	25	16	382	2.96	1.82×10^6	1.81×10^6(99.5)	
3.	-3-^{14}C	(a)	12.5	4.2	483	1.47	3.57×10^5	3.55×10^5(99.4)	8.92×10^3 (2.5)
		(b)	25	8.4	268	3.45	3.02×10^6	2.96×10^6(98.0)	
4.	Malonic acid -2-^{14}C	(a)	100	23	464	3.40	6.86×10^6	6.66×10^6(97.0)	6.45×10^6(94.0)

Feeding period: (a) 15 hr, (b) 50 hr.
a) Figures in parentheses show % ratio to the specific activity of mangiferin.

As shown in Tables I and II, phenylalanine-1-^{14}C, -2-^{14}C and -3-^{14}C, cinnamic acid-3-^{14}C and *p*-coumaric acid-2-^{14}C and -(ring-3,5-T_2) were efficiently incorporated into **1**, and in these cases the radioactivity was localized in the xanthone moiety of **1** (Expts. 1—6). In contrast, incorporation of benzoic acid-, *p*-hydroxybenzoic acid- and protocatechuic acid-(carboxyl-^{14}C) and *p*-hydroxybenzoic acid-3,5-T_2 into **1** was much lower than that of the above C_6—C_3 precursors, and in these cases only about 30% of the label was distributed in the xanthone

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12) M. Fujita and T. Inoue, *Yakugaku Zasshi*, **99**, 165 (1979).

Table II. Incorporations of Labelled Cinnamic Acid and Benzoic Acid Derivatives into Mangiferin and Isomangiferin

Expt.	Precursors	Amount fed (μCi)	Amount fed (×10⁻¹ mg)	C-Glucosylxanthones	Yield (mg)	Incorporation (%)	Sp. act. (dpm/mM) C-Glucosylxanthones	Sp. act. (dpm/mM) Aglycone[a] (tetraacetate)
5.	Cinnamic acid-3-^{14}C	50	1.5	Mangiferin	458	3.09	3.16×10^6	3.10×10^6 (98.1)
				Isomangiferin	69	0.26	1.78×10^6	1.72×10^6 (96.6)
6.	*p*-Coumaric acid -2-^{14}C	3.26	30	Mangiferin	323	1.19	1.13×10^5	1.14×10^5(100.9)
				Isomangiferin	52	0.10	5.98×10^4	5.80×10^4 (97.0)
7.	*p*-Coumaric acid -(ring-3,5-T_2)	5.40	30	Mangiferin	302	0.52	8.80×10^4	
8.	Benzoic acid -(carboxyl-^{14}C)	50	1.1	Mangiferin	483	0.02	1.87×10^4	3.77×10^3 (20.2)
9.	*p*-Hydroxybenzoic acid-(carboxyl-^{14}C)	100	2.7	Mangiferin	301	0.02	5.64×10^4	1.51×10^4 (26.8)
				Isomangiferin	48	0.001	2.19×10^4	6.67×10^3 (30.5)
10.	*p*-Hydroxybenzoic acid-3,5-T_2	1.20	50	Mangiferin	344	0.05	4.60×10^3	
11.	Protocatechuic acid -(carboxyl-^{14}C)	1.78	50	Mangiferin	288	0.04	2.20×10^3	7.28×10^2 (33.1)
				Isomangiferin	40	0.003	1.03×10^3	

Feeding period: 50 hr.
a) Figures in parentheses show % ratios to the specific activity of the *C*-glucosylxanthones.

moiety of **1** and the remainder in the sugar moiety (Expts. 8, 9 and 11). Cinnamic acid-3-^{14}C and *p*-coumaric acid-2-^{14}C were also incorporated into isomangiferin (**2**), but *p*-hydroxybenzoic acid- and protocatechuic acid-(carboxyl-^{14}C) were not.

If phenylalanine was utilized for the formation of the xanthone nucleus of **1** without loss of two carbon fragments, the C-1 and C-2 carbons of phenylalanine would be incorporated into the phloroglucinol ring of the aglycone (**3**) and the C-3 carbon would not. Thus, mangiferin (**1**) obtained by feeding with labelled phenylalanine was directly degraded to phloroglucinol by potash fusion. As shown in Table I, the radioactivity was almost exclusively present in the phloroglucinol ring of **1** when phenylalanine-1-^{14}C or -2-^{14}C was fed. On the other hand, the label of phenylalanine-3-^{14}C was essentially not incorporated into the phloroglucinol moiety of **1**.

These findings suggest that C_6—C_3 compounds, and not C_6—C_1 ones, could be direct precursors for the biosynthesis of **1** and **2**.

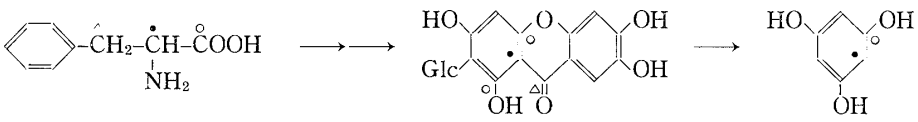


Chart 2

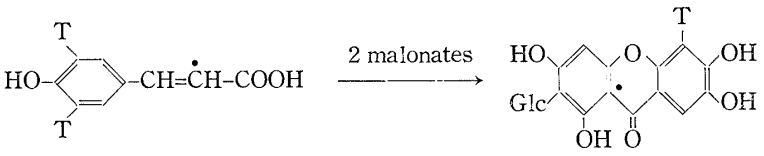
In order to obtain conclusive evidence for the participation of a C_6—C_3 unit, a mixture of *p*-coumaric acid-(ring-3,5-T_2) and -2-^{14}C was fed to the plants and the T/^{14}C ratio of mangiferin (**1**) isolated was compared with that of the precursor fed (Table III). Considering the loss (1/2) of T on arylhydroxylation during the biosynthesis of **1**, the results indicated that the doubly labelled *p*-coumaric acid was incorporated into **1** essentially without change of the T/^{14}C ratio. Malonic acid-2-^{14}C was also well incorporated into **1**, as expected, and the radioactivity was largely present in the phloroglucinol ring of **1** (Expt. 7).

All the feeding experiments indicate that the aglycone (**3**) of **1** and **2** is biosynthesized by the cyclization of an intermediate derived from *p*-coumarate and two malonates. This represents a new route for xanthone biosynthesis, which is different from that of gentisin in *Gentiana lutea*.

Table III. Ratio of T and ^{14}C Activities in the Precursor and Mangiferin after Feeding T- and ^{14}C-Labelled *p*-Coumaric Acid

Expt.		T/^{14}C[a]	Sp. act. (dpm/mM)		Incorp. (%)		T/^{14}C[b]
			T	^{14}C	T	^{14}C	
12.	(a)	1.68	7.39×10^4	9.57×10^4	0.52	1.11	1.54
	(b)	1.65	7.66×10^4	1.03×10^5	0.52	1.16	1.49

Precursor: A mixture of *p*-coumaric acid-[ring-3,5-T_2] (6.56×10^8 dpm/mM) +*p*-coumaric acid-2-^{14}C (3.96×10^8 dpm/mM), 3 mg each.
Feeding period: (a) 50 hr, (b) 70 hr.
a) Ratio in the precursor.
b) Ratio in mangiferin: This ratio is corrected for loss of T on arylhydroxylation during biosynthesis.



It has been suggested that the aglycone (**3**) is probably formed by oxidative coupling of a benzophenone.[13] Mangiferin (**1**) has a characteristic distribution in plants[3a,7] and frequently occurs together with *C*-glucosylflavones.[14] This suggests that the biosynthesis of **1** is related to that of flavonoids. On the other hand, O'Donovan *et al.*[15] reported that lobeline was biosynthesized from benzoylacetic acid (**4**) *via* 3-hydroxy-3-phenylpropionic acid (**5**), which was derived from cinnamic acid. Recently Herbert *et al.*[16] demonstrated that a phenanthroindolizidine alkaloid, tylophorinine, is biosynthesized from *p*-hydroxybenzoylacetic acid (**6**) in the same way as lobeline. 3-Hydroxy-3-phenylpropionic acid and benzoylacetic acid derivatives have also been considered as intermediates in the metabolic pathway of cinnamic acid derivatives to the corresponding benzoic acids[17] and acetophenones.[18] In particular, it should be noted that acetophenone derivatives[19] were isolated from *Iris florentina* along with a benzophenone, iriflophenone (**7**),[20] and *C*-glucosylxanthones.[21]

Therefore, the alternative routes (a) and (b) are suggested for the formation of a benzophenone in mangiferin biosynthesis; (a) *p*-coumarate might be condensed with two malonates, instead of three malonates as in flavonoid biosynthesis,[22] to yield of C-13 precursor (**8**), followed by oxidation to a β-triketo ester (**9**) and cyclization to a benzophenone or (b) the benzoylacetic acid (**6**) derived from *p*-coumarate might be condensed with two malonates

13) H.D. Locksley, I. Moore, and F. Scheinmann, *Tetrahedron*, **23**, 2229 (1967), and references cited therein.
14) E.C. Bate-Smith, *Mem. Soc. Bot. Fr.*, **1965**, 16; T. Swain, *ibid.*, **1965**, 184; E.C. Bate-Smith and T. Swain, *Lloydia*, **28**, 313 (1965); T. Tomimori and M. Komatsu, *Yakugaku Zasshi*, **89**, 1276 (1969).
15) D.G. O'Donovan, D.J. Long, E. Forde, and P. Geary, *J. Chem. Soc. Perkin I*, **1975**, 415.
16) R.B. Herbert, F.B. Jackson, and I.T. Nicolson, *Chem. Commun.*, **1976**, 865.
17) M.H. Zenk, *Z. Naturforsch.*, **19b**, 83 (1964); M.H. Zenk and G. Müller, *ibid.*, **19b**, 398 (1964); H. Grisebach and K.O. Vollmer, *ibid.*, **18b**, 753 (1963); D.J. Bennet and G.W. Kirby, *J. Chem. Soc.* (*C*), **1968**, 442; M.H. Zenk, "Pharmacognosy and Phytochemistry," ed. by H. Wagner and L. Hörhammer, Springer-Verlag, Berlin, Heidelberg, New York, 1971, pp. 314—346; C.J. French, C.P. Vance, and G.H.N. Towers, *Phytochemistry*, **15**, 564 (1976), and references cited therein.
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19) M. Fujita and T. Inoue, unpublished work.
20) M. Arisawa, N. Morita, Y. Kondo, and T. Takemoto, *Chem. Pharm. Bull.*, **21**, 2323 (1973).
21) M. Arisawa, N. Morita, Y. Kondo, and T. Takemoto, *Chem. Pharm. Bull.*, **21**, 2562 (1973).
22) K. Hahlblock and H. Grisebach, "The Flavonoids," ed. by J.B. Harbone, T.J. Mabry and H. Mabry, Chapman and Hall Ltd., London, 1975, pp. 866—915; G. Hrazdina, F. Kreuzaler, K. Hahlblock, and H. Grisebach, *Arch. Biochem. Biophys.*, **175**, 392 (1976); F. Kreuzaler and K. Hahlblock, *Europ. J. Biochem.*, **56**, 205 (1975); N.A.M. Saleh, H. Fritsch, and H. Grisebach, *Phytochemistry*, **17**, 183 (1978).

to yield **9**, followed by cyclization to a benzophenone. Studies to determine whether the xanthone nucleous of mangiferin (**1**) is biosynthesized *via* either route (a) or (b) are in progress.

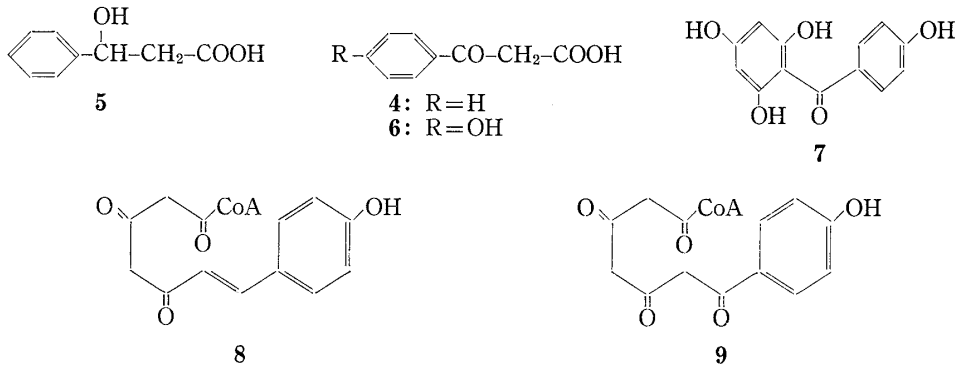

Chart 3

Experimental

Phenylalanine-1-^{14}C (61 mCi/mM), -2-^{14}C (25 mCi/mM) and benzoic acid-(carboxyl-^{14}C) (57.8 mCi/mM) were purchased from the Radiochemical Centre, Amersham, phenylalanine-3-^{14}C (50 mCi/mM), cinnamic acid-3-^{14}C (58 mCi/mM), *p*-hydroxybenzoic acid-(carboxyl-^{14}C) (55 mCi/mM) and veratric acid-(carboxyl-^{14}C) (59 mCi/mM) from Commissariat A L'Energie Atomique and malonic acid-2-^{14}C (45.9 mCi/mM) from New England Nuclear Corp. All radioactive samples were counted with an Aloka LSC-602 liquid scintillation counter, in a POP-POPOP-naphthalene-dioxane scintillator solution. The purity of synthesized radioactive compounds was examined by thin-layer radiochromatography (Aloka JTC-201).

***p*-Coumaric Acid-(ring-3,5-T_2)**[11]——*p*-Hydroxybenzaldehyde-3,5-T_2[9] (sp. act. 1.87×10^9 dpm/mM, 70 mg), *p*-hydroxybenzaldehyde (125 mg) and malonic acid (333 mg) were dissolved in pyridine (0.4 ml), and aniline (0.01 ml) was added to the solution. The mixture was heated at 65° for 5 hr, then diluted with H_2O (7 ml) and allowed to stand overnight in the icebox. The resulting precipitates were washed with cold water and recrystallized from aq. MeOH to give colorless crystals (157 mg), mp 209—210°. Sp. act. 6.56×10^8 dpm/mM.

***p*-Hydroxybenzoic Acid-3,5-T_2**[10]——*p*-Hydroxybenzaldehyde-3,5-T_2 (33 mg), *p*-hydroxybenzaldehyde (90 mg) and NaOH (240 mg) were dissolved in H_2O (2 ml), and $AgNO_3$ (170 mg) in H_2O (0.25 ml) was added to the solution at 50° with stirring. The mixture was stirred for 5 min at 50° and then for 25 min at room temperature, and filtered. The precipitates were washed with H_2O, and the combined filterate and washings were acidified with SO_2 gas. After a day, the resulting colorless crystals (102 mg) were recrystallized from H_2O, mp 213—214°. Sp. act. 2.12×10^8 dpm/mM.

Protocatechuic Acid-(carboxyl-^{14}C)——Veratric acid-(carboxyl-^{14}C) (0.1 mCi, 0.34 mg) was diluted with veratric acid (150 mg) and refluxed with HI (20 ml) for 4 hr. The reaction mixture was treated in the usual way, and the crude product was diluted with carrier (50 mg) and recrystallized from H_2O to give almost colorless crystals (100 mg), mp 196—199°. Sp. act. 1.22×10^8 dpm/mM.

Feeding Experiments——Labelled cinnamic acid, *p*-coumaric acid and benzoic acid were dissolved in a minimum amount of 0.01 N NaOH, and the other compounds were dissolved in H_2O. The aerial parts of *A. asphodeloides* were cut from the rhizomes in the flowering period and immersed in the above precursor solutions. The solution was almost completely within 2—3 hr, and then H_2O was added repeatedly in order to ensure that the remaining precursor was absorbed. The total feeding time was 15—70 hr.

Isolation of Mangiferin (1) and Isomangiferin (2)——After feeding the plants were cut into pieces, dried at 60° and extracted repeatedly with hot MeOH. The extracts were concentrated to dryness, the residue was dissolved in hot H_2O and the insoluble material was filtered off. The filtrate was washed with Et_2O, and the aqueous solution was passed through a column of polyamide (Woelm) then washed with a large amount of H_2O. The adsorbed material was eluted with MeOH, and the eluant was concentrated to small volume then left overnight. The crude mangiferin (**1**) was obtained as a pale yellow crystalline powder (about 3.5% yield from the dried plants) which was recrystallized from aq. MeOH to constant activity. The filtrate after removal of crude **1** was chromatographed on cellulose using 3% AcOH as an eluent. After the elution of **1**, crude isomangiferin (**2**) was obtained by elution with 10% AcOH. The crude **2** was purified by polyamide column chromatography and elution with MeOH gave **2**, which was recrystallized from aq. MeOH to give a pale yellow crystalline powder (about 0.5% yield from the dried plants).

Treatment of 1 and 2 with HI——A solution of **1** or **2** (50 mg) in phenol (250 mg) was refluxed with HI (d=1.7, 1 ml) for 5 hr at 150°, then the reaction mixture was poured into satur. $NaHSO_3$ solution, and the

resulting precipitates were collected, washed with H_2O and dried. The pale brown product was heated with Ac_2O (25 ml) and AcONa (100 mg) for 2 hr, then the reaction mixture was poured into H_2O, and the resulting precipitates were collected and washed with H_2O. The crude acetate was chromatographed on silica gel using C_6H_6–EtOAc (4: 1) as an eluent, followed by recrystallization from MeOH to give 1,3,6,7-tetraacetoxyxanthone as colorless crystals (16 mg), mp 189—191°.

Alkali Fusion of 1——Radioactive mangiferin (**1**) was diluted about 10 times with carrier and degraded as follows. **1** (2.4 g) was gradually added to a mixture of fused KOH and NaOH (10 g each), and the whole was heated at 250—270° under an N_2 stream for 3 hr. The reaction mixture was cooled and acidified with dil.HCl under cooling. The aqueous solution was saturated with NaCl and extracted with ether (5×500 ml). The ethereal layer was concentrated to about 500 ml and washed with $NaHCO_3$ solution (3×80 ml). The ethereal layer was extracted with 5% NaOH solution (3×80 ml), and the aqueous layer was acidified with dil. HCl, saturated with NaCl and extracted with ether (5×300 ml). The ethereal layer was washed with a small amount of H_2O, dried over anhyd. $MgSO_4$ and evaporated to dryness *in vacuo*. The residue was separated by preparative thin-layer chromatography (Merck, Silica gel GF_{254}) using AcOEt as an eluent, followed by sublimation *in vacuo* to give phloroglucinol as a white powder (12 mg), mp 214—215°.

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