The *Para*-O-Methylation of Apigenin to Acacetin by Cell-Free Extracts of *Robinia pseudoacacia* L.

Gary Kuroki and Jonathan E. Poulton

Department of Botany, University of Iowa, Iowa City, Iowa 52242

Z. Naturforsch. **36 c**, 916–920 (1981); received July 6, 1981

Robinia pseudoacacia, Biosynthesis, Flavonoids, *Para*-O-Methyltransferase, Acacetin

Crude extracts from young *Robinia pseudoacacia* seedlings, shoots, and callus tissue catalyze the *para*-O-methylation of apigenin to acacetin using S-adenosyl-L-methionine as methyl donor. Optimum activity was exhibited at pH 9.0, and Mg^{2+} was not required for maximum activity. EDTA (10 mM) did not affect the reaction rate, but 47% inhibition was observed with SAH (100 μM). β-Mercaptoethanol (5 mM) was required in the homogenization medium for optimum O-methyltransferase activity. Apigenin (K_m, 50 μM) was the best substrate, but significant activity was shown towards caffeic acid, 5-hydroxyferulic acid, naringenin, and quercetin. *Para*-coumaric, ferulic, and sinapic acids were not methylated. The K_m for S-adenosyl-L-methionine was 31 μM. Our demonstration of a *para*-O-methyltransferase activity methylating apigenin, but not *para*-coumaric acid, strongly supports the conclusion that the B-ring methylation pattern of acacetin is determined at the C_{15}-level in *Robinia pseudoacacia*.

Introduction

Flavonoids possessing methoxyl groups at the 3′- and/or 5′-positions of the B-ring are widespread in Nature, and their enzymatic biosynthesis is well documented [1]. By contrast, flavonoids bearing a 4′-methoxyl group are relatively rare, and virtually nothing is known about the enzymes involved in the biosynthesis of these pigments [2].

Since black locust (*Robinia pseudoacacia* L.) seedlings rapidly accumulate acacetin (4′-methoxy-5,7-dihydroxyflavone) glycosides in the first week following germination, they provide an excellent system with which to investigate the biosynthesis of 4′-methoxylated flavonoids. We wish to report a novel *para*-O-methyltransferase activity in crude *Robinia* extracts, which methylates apigenin at the 4′-position, yielding acacetin. This activity is discussed in relation to earlier isotopic studies designed to establish whether the B-ring substitution pattern of acacetin is determined at the C_9- or C_{15}-level [3].

Abbreviations: SAM, S-adenosyl-L-methionine; EGME, ethylene glycol monomethyl ether; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; EDTA, ethylenediaminetetraacetic acid; SAH, S-adenosyl-L-homocysteine.

Reprint requests to J. E. Poulton.

0341-0382/81/1100-0916 $ 01.00/0

Materials and Methods

Chemicals

S-Adenosyl-L-[$^{14}CH_3$]methionine (57.9 mCi/mmol) was purchased from New England Nuclear, Boston, Mass., and diluted with unlabelled SAM to a specific activity of 1.1 mCi/mmol. S-Adenosyl-L-homcysteine was purchased from Sigma Chemical Co., St. Louis, Mo. Acacetin glycosides were isolated from *Robinia* seedlings and hydrolyzed using 1 M HCl at 90 °C to obtain acacetin, which was identified by standard spectral methods [4]. Phenolic substrates were available from our laboratory collection. Clorox was purchased from the Clorox Co., Oakland, Ca.

Plant Materials

Black locust (*Robinia pseudoacacia* L.) seeds were collected from the University of Iowa campus. The seeds were sterilized in 10% Clorox for 0.5 h, washed three times with sterile water, and scarified with a razor blade. The seeds were placed in sterile aerated water for 48 h and sown in sterile soil. The germinated seeds were placed in a growth chamber maintained at 24 °C and under a 16:8 h (light:dark) photoperiod. Cell cultures were established from *Robinia* cotyledons by placing the sterile explants on Murashige and Skoog media [5] containing 10 mg/l naphthalene acetic acid, and 0.1 mg/l kinetin. Callus tissue was maintained at 25 °C under a 16:8 h

(light:dark) photoperiod and subcultured every 4 weeks.

Enzyme Purification

Unless otherwise indicated, all stages were carried out at 4 °C. *Robinia* seedlings (10–12 days old; weighing approximately 5.5 g) were homogenized with 30 ml of buffer I and 2.5 g of quartz sand in a mortar. The homogenate was filtered through 2 layers of cheesecloth and centrifuged at $12000 \times g$ for 15 min. The supernatant liquid was brought to 80% saturation by addition of solid $(NH_4)_2SO_4$ over a period of 20 min. Small volumes of dilute aqueous KOH were added, where necessary, to maintain the pH at 7.3. The mixture was allowed to stand for an additional 40 min at 0 °C and was then centrifuged at $17600 \times g$ for 15 min. The pellet was resuspended in 3 ml of buffer II and centrifuged for 5 min at $17600 \times g$ to remove undissolved material. An aliquot (2.5 ml) of the supernatant liquid was chromatographed on a Sephadex G-25 column (1.5 × 8.3 cm), which had been pre-equilibrated with buffer II. Elution was carried out with this buffer, and the eluate was collected for assay of methyltransferase activity. Aliquots (0.5 ml) of this enzyme preparation were stored at −20 °C and thawed as required.

Buffer solutions

The following buffer solutions were used: (I) 0.1 M potassium phosphate buffer, pH 7.5, containing 5 mM β-mercaptoethanol; (II) 20 mM potassium phosphate buffer, pH 7.5, containing 5 mM β-mercaptoethanol.

O-Methyltransferase assay

The standard assay mixture for O-methyltransferase activity contained 150 nmol phenolic substrate (dissolved in 10 µl EGME), 26 nmol S-adenosyl-L-[$^{14}CH_3$]methionine (containing 41 nCi), 50 nmol $MgCl_2$, 10 µmol Tris-HCl buffer, pH 9.0, and up to 0.88 mg protein in a total volume of 0.15 ml. After incubation at 30 °C for 45 or 60 min, the reaction was terminated by adding 50 µl EGME and placing the reaction mixture on ice. After addition of 20 µl glacial acetic acid, the radioactive product was extracted into 0.5 ml of ethyl acetate [6]. Aliquots (0.3 ml) of the organic layer were mixed with 5 ml of Andersons scintillation fluid (0.3% PPO and 0.02% POPOP in xylene-Triton X-114 (3:1, by vol.)) and the radioactivity measured in a Beckman LS-100 C scintillation counter. Control reaction vessels, in which the phenolic substrates were omitted, were included in all assays performed.

Chromatographic identification of reaction product

Acacetin was identified as the product of apigenin methylation by co-chromatography with an authentic sample on Whatman 3 MM paper using the following solvent systems: (I) 1% HCl, (II) 15% acetic acid, (III) 30% methanol, (IV) ethyl acetate-pyridine-H_2O (12:5:4, by vol.). Additional confirmation was provided by co-chromatography on silica gel IB-F thin layer sheets (J. T. Baker Chem. Co., N.J.) with the following solvent systems: (V) benzene-dioxane-acetic acid (90:25:4, by vol.), (VI) toluene-ethyl acetate-acetic acid (9:2:0.5, by vol.).

Protein estimation

Protein was determined by the Lowry method, as modified by Leggett Bailey [7], after precipitation from solution by 5% (w/v) trichloroacetic acid; crystalline bovine serum albumin was used as the standard.

Results and Discussion

A central problem in flavonoid biochemistry which still remains unresolved is to establish when the B-ring substitution pattern is determined. Black locust (*Robinia pseudoacacia*) shoots contain glycosides not only of the common flavonoids apigenin, kaempferol and quercetin but also of acacetin, which exhibits the more unusual 4′-methoxylated B-ring [3]. The predominant acacetin glycosides are acaciin (acacetin-7-rutinoside) [8] and acacetin 7-xylosylrhamnosylglucoside [9]. The time at which the B-ring substitution pattern of acacetin is established was investigated by Ebel, Barz and Grisebach [3] using radiotracer techniques. When older *Robinia* leaves were incubated for 64 h with *p*-methoxycinnamic acid-[β-^{14}C-methyl-^{14}C-methyl-T], the T/^{14}C ratio in acacetin was only 23% of that of the precursor, suggesting active demethylation of *p*-methoxycinnamate in such leaves. By contrast, when younger leaves were fed *p*-methoxycinnamic acid-[β-^{14}C-methyl-T] for shorter periods of time, the

T/^{14}C ratio in acacetin was almost the same as that of the precursor. Although this constitutes strong evidence for the incorporation of *p*-methoxycinnamic acid as an intact unit into acacetin, the extent of incorporation was only 0.01 – 0.02%.

We wished to complement these radiotracer data by enzymological studies, especially since little is known about the *para*-O-methylation of either *p*-coumaric acid [10] or flavonoids [11]. As climatic conditions preclude the full-year availability of *Robinia* shoots, additional experimental systems were investigated. *Robinia* seeds possess no acacetin glycosides. However, in the first week following germination, the young seedlings rapidly accumulate 4 acacetin glycosides (nature of sugar moieties as yet unidentified) and therefore provide an excellent system for identifying enzymes involved in the biosynthesis of the flavonoids. In addition, callus cultures were established from *Robinia* seedling cotyledons as described in the Methods section. We investigated the ability of extracts from *Robinia* seedlings, shoots, and callus tissue to methylate phenolic substrates, using SAM as methyl donor. All attempts to demonstrate the methylation of *p*-coumaric acid to *p*-methoxycinnamic acid were unsuccessful. Instead, the ready methylation of apigenin to its 4′-O-methyl ether acacetin was detected and is the subject of this publication.

A crude protein preparation was obtained from young *Robinia* seedlings by homogenization, ammonium sulphate precipitation and gel filtration, as described in the Methods section. β-Mercaptoethanol (5 mM) was routinely added to the homogenization medium, since apigenin 4′-O-methyltransferase activities were reduced to 20% of control values when homogenization was undertaken in its absence (Table I). This rate could be stimulated by only a further 37% when an equivalent amount of β-mercaptoethanol was subsequently added to the assay medium.

Table I. The effect of β-mercaptoethanol on O-methyltransferase activity.

Treatment of seedlings	Specific activity [cpm/h/mg protein]		
	Apigenin	Quercetin	Caffeic acid
Homogenized in phosphate buffer containing 5 mM β-mercaptoethanol	9868	8061	4103
Homogenized in phosphate buffer lacking β-mercaptoethanol	2010	2010	2434
Homogenized in phosphate buffer lacking β-mercaptoethanol; β-mercaptoethanol subsequently added to the assay	5635	6447	5353

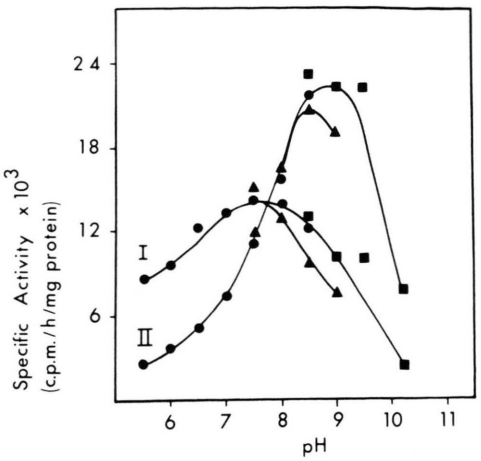

Fig. 1. Effect of pH on the O-methyltransferase activity towards apigenin (II) and caffeic acid (I). The phenolic substrate and SAM were incubated with 0.69 mg of protein as described under Materials and Methods, using 67 mM concentrations of the following buffers: K_2HPO_4-KH_2PO_4 (●–●), Tris-HCl (▲–▲), and glycine-NaOH (■–■).

The optimum pH for the methylation of apigenin was determined using several different buffers (Fig. 1). Apigenin methylation proceeded most rapidly at pH 9.0, but over 50% of this maximum rate was realized over the range between pH 7.5 and 10. A similarly high pH optimum was reported for the isoflavone-specific *para*-O-methyltransferase from *Cicer arietinum* cell cultures [11] and for the 3,4-dihydric phenol 3-O-methyltransferases from parsley [12] and soybean cell cultures [13], which prefer flavonoid substrates. The rate of apigenin methylation at pH 9.0, catalysed by 0.66 mg of the partially purified enzyme was linear for at least 60 min. The extent of methylation at this pH after incubation for 45 min was proportional to the protein concentration up to at least 0.66 mg of the enzyme preparation. The addition of Mg^{2+} ions at concentrations from 0.18 to 10 mM had no significant effect on the rate of apigenin methylation (Table II). Furthermore, EDTA, supplied at concentrations of 1 mM

Table II. Influence of Mg^{2+} ions and EDTA on O-methyltransferase activity*.

Substrate	$MgCl_2$ concentration [mM]	EDTA concentration [mM]	Specific activity [cpm/h/mg protein]
apigenin	0	0	25 653
	0.18	0	25 179
	0.35	0	22 206
	2.0	0	24 505
	10	0	27 891
	0	1.0	30 068
	0	10	25 845
quercetin	0	0	11 880
	0.18	0	11 393
	0.35	0	13 179
	2.0	0	15 350
	10	0	16 100
	0	1.0	8 026
	0	10	9 251
caffeic acid	0	0	4 983
	0.18	0	4 405
	0.35	0	4 540
	2.0	0	5 291
	10	0	5 695
	0	1.0	2 950
	0	10	2 680

* Assayed in 67 mM Tris-HCl buffer, pH 9.0.

and 10 mM, did not inhibit the enzyme. Similar behaviour towards Mg^{2+} ions and EDTA was observed with the isoflavone 4′-O-methyltransferase from chick peas [11].

The substrate specificity of the enzyme preparation was tested in the presence of 0.33 mM $MgCl_2$ at pH 9.0 (Fig. 2). Table III indicates that apigenin was the best substrate. The apparent K_m for apigenin was 50 μM, when assayed in the presence of a saturating concentration of SAM (171 μM). The apparent K_m for SAM, assayed under identical conditions in the presence of 1 mM apigenin, was 31 μM. Among other monophenolic substrates tested, only naringenin (5,7,4′-trihydroxyflavanone) was methylated, exhibiting a rate of ~ 47% that observed with apigenin. As far as we are aware, this is the first report within the literature of the O-methylation of flavones and flavanones at the 4′-position. By contrast, *p*-coumaric acid was not methylated by these preparations. Several diphenolic substrates were also methylated significantly including quercetin, caffeic acid and 5-hydroxyferulic acid, but the position of methylation was not identified. Review of the literature strongly suggests that the methylations of these diphenolic substrates would most likely be carried out by enzymes distinct from the apigenin 4′-O-methyltransferase [1]. Several lines of circumstantial evidence gathered in this study favour this hypothesis: (i) In contrast to the high pH optimum obtained with apigenin, the maximum rate of caffeic acid methylation was observed around pH 7.5, a value characteristic for several *meta*-specific O-methyltransferases involved in lignin biosynthesis (Fig. 1); (ii) In contrast to apigenin 4′-O-methyltransferase activity, both caffeic acid- and quercetin-methyltransferase activities were stimulated by 10 mM $MgCl_2$ by 14% and 36%, respectively (Table II). Furthermore, EDTA (10 mM) inhibited the maximum rate of methylation of caffeic acid and quercetin by 53% and 43%, respectively; (iii) Methyltransferase activities towards apigenin, caffeic acid and quercetin showed different behaviour upon storage at either 4 °C or − 20 °C (data not shown); (iv) Slight differences were ob-

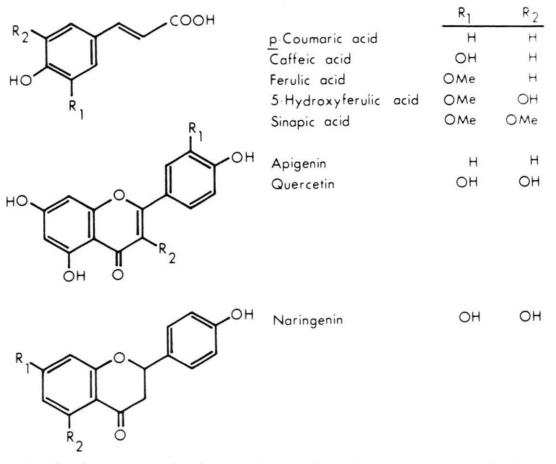

Fig. 2. Structural formulae of substrates used in the O-methyltransferase assays.

Table III. Substrate specificity of *Robinia* O-methyltransferase preparation. O-Methyltransferase activity was assayed at pH 9.0 as described in the Methods section. Substrate concentration was 100 μM in each assay.

Substrate	Specific activity [cpm/h/mg protein]
apigenin	25 410
quercetin	17 696
naringenin	11 888
5-hydroxyferulic acid	8 057
caffeic acid	7 514
p-coumaric acid	0
ferulic acid	0
sinapic acid	0

Table IV. Inhibition of O-methyltransferase activity by S-adenosyl-L-homocysteine.

Substrate	SAH concentration [μM]	Specific activity [cpm/h/mg protein]	% of control activity
apigenin	0	19 174	100
	20	16 159	84
	50	14 349	75
	100	10 166	53
	300	4 749	25
quercetin	0	10 240	100
	20	9 923	97
	50	8 339	81
	100	6 066	59
	300	2 955	29
caffeic acid	0	4 896	100
	20	3 923	80
	50	3 426	70
	100	2 757	56
	300	1 498	30

served in the extent to which SAH might inhibit the methyltransferase activity towards these three substrates (Table IV).

The goal of future experimentation is to resolve this apparent mixture of O-methyltransferases, with special interest for the isolation and characterization of the flavone 4′-O-methyltransferase. In recent years, it has been clearly demonstrated that most, if not all, trans-methylation reactions are potently inhibited by low concentrations of the reaction product SAH [1]. This feature allowed the resolution of methyltransferases by affinity chromatography with SAH as ligand [14, 15]. To investigate the possibility that the *Robinia* O-methyltransferases might be purified by SAH-affinity chromatography, we determined the effect of various concentrations on SAH upon the methylation rate with key substrates (Table IV). Significant inhibition of all three methyltransferase activities was observed.

Apigenin 4′-O-methyltransferase activity was not restricted only to young black locust seedlings (specific activity: 6.7 nmol acacetin produced/h/mg protein). Comparable activities were also demonstrated in extracts from young *Robinia* shoots (3.7 nmol acacetin/h/mg protein) and callus cultures (23 nmol acacetin/h/mg protein). We believe that the current demonstration of a flavone 4′-O-methyltransferase activity which methylates apigenin to acacetin, together with the failure of these extracts to methylate *p*-coumaric acid, strongly support the "substitution hypothesis" of Grisebach [2], according to which the methylation pattern of acacetin should be established at the C_{15}-level, rather than at the C_9-level as was previously predicted by radiotracer studies [3].

[1] J. E. Poulton, in: The Biochemistry of Plants: A comprehensive treatise, (E. E. Conn and P. K. Stumpf, eds.), **Vol. VII,** pp. 667–723, Academic Press, New York 1981.
[2] K. Hahlbrock and H. Grisebach, in: The Flavonoids, (J. B. Harbone, T. J. Mabry, and H. Mabry, eds.), pp. 866–915, Chapman and Hall, London 1975.
[3] J. Ebel, W. Barz, and H. Grisebach, Phytochemistry **9,** 1529–1534 (1970).
[4] T. J. Mabry, K. R. Markham, and M. B. Thomas, in: The Systematic Identification of Flavonoids, pp. 41–57, Springer-Verlag, New York 1970.
[5] T. Murashige and F. Skoog, Physiol. Plant. **15,** 473–497 (1962).
[6] J. E. Poulton, H. Grisebach, J. Ebel, B. Schaller-Hekeler, and K. Hahlbrock, Arch. Biochem. Biophys. **173,** 301–305 (1976).
[7] J. Leggett Bailey, in: Techniques in Protein Chemistry, pp. 293–294, Elsevier Publishing Co., Amsterdam, 1962.
[8] S. Hattori, Acta Phytochim. Japan **2,** 99–101 (1925).
[9] K. Freudenberg and L. Hartmann, Liebigs Ann. Chem. **587,** 207–212 (1954).
[10] K. Kaneko, Chem. Pharm. Bull. **10,** 1085–1087 (1962).
[11] H. Wengenmayer, J. Ebel, and H. Grisebach, Eur. J. Biochem. **50,** 135–143 (1974).
[12] J. Ebel, K. Hahlbrock, and H. Grisebach, Biochem. Biophys. Acta **268,** 313–326 (1972).
[13] J. E. Poulton, K. Hahlbrock, and H. Grisebach, Arch. Biochem. Biophys. **180,** 543–549 (1977).
[14] J. P. G. Mack and M. B. Slaytor, J. Chromatogr. **157,** 153–159 (1978).
[15] S. K. Sharma and S. A. Brown, J. Chromatogr. **157,** 427–431 (1978).