Stepwise Methylation of Quercetin by Cell-Free Extracts of Citrus Tissues

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The cell-free extracts of peel, root, or callus tissues of citrus catalyzed the stepwise O-methylation of quercetin to rhamnetin, isorhamnetin and rhamnazin. Both rhamnetin and isorhamnetin were further methylated to rhamnazin and possibly to a trimethyl ether derivative of quercetin. The results seem to indicate the existence of both *meta* and *para* directing enzymes that are involved in the biosynthesis of methylated flavonoids in citrus tissues.

O-Methylated flavonoids are known for their wide spread occurrence in the plant kingdom [1]. Whereas the *in vitro* O-methylation of ring-B of flavonoids has been reported with a number of enzyme preparations [2–5] that of ring-A, on the other hand, has so far received little attention [6–9]. Recently, however, Poulton *et al.* [4] reported the efficient methylation of texasin (6,7-dihydroxy-4'-methoxyisoflavone) by a purified soybean O-methyltransferase, though the exact position of methylation remains to be determined. We wish to report here, for the first time, the stepwise O-methylation of quercetin, by cell-free extracts of citrus tissues, to rhamnetin, isorhamnetin and rhamnazin.

The source of enzyme preparation was either (a) the peel tissue of immature calamondin *(Citrus mitis)* peel, (b) a callus tissue culture that was initiated from calamondin seed, or (c) the root system of 6-week old 'Sunkist' orange seedlings. All procedures were conducted at 2–4 °C. Fresh or frozen tissues were mixed with sand and Polyclar AT and homogenized with 0.1 M Tris-HCl buffer, pH 7.8, containing 1 mM EDTA and 14 mM β-mercaptoethanol. The supernatant obtained after centrifugation (20 min, 15000 *xg*) was fractionated with solid ammonium sulphate and the protein which precipitated between 30–70% saturation was collected by centrifugation and dissolved in 50 mM of the same buffer. It was desalted on Sephadex G-25 column and was directly used as the enzyme source.

The standard assay mixture of O-methyltransferase consisted of 40–60 nmol of the phenolic substrate (dissolved in DMSO), 10 nmol S-[$^{14}CH_3$]-adenosyl-L-methionine (New England Nuclear) containing 55,000 cpm and the enzyme protein (in Tris-HCl buffer, pH 7.8) in a total volume of 200 μl. The enzyme reaction was allowed to proceed for 30 min at 30 °C and was stopped by the addition of 20 μl of 6 N HCl. Assays were conducted in duplicates and the reaction products were extracted with benzene-ethyl acetate (1:1). The products of one assay were counted for total radioactivity by liquid scintillation. Those of the other assay were co-chromatographed with reference compounds on silica plates using benzene-pyridine-formic acid (36:18:5 or 86:19:5) as solvent system [10] and then autoradiographed. In most cases two-dimentional chromatography, using chloroform-methanol (15:1) for the second direction, was performed in order to verify the identity of the reaction products.

The results given in Table I show that the cell-free extracts of peel, root or callus tissues of citrus

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Substrate	Substitution		Source [b]	Enzyme activity [c]		
	OH	OMe		Root	peel	callus
1. Quercetin	3, 5, 7, 3', 4'	—	M	10,050	14,100	27,700
2. Isorhamnetin	3, 5, 7, 4'	3'	R	2,960	4,150	5,540
3. Rhamnetin	3, 5, 3', 4'	7	R	4,320	6,770	9,140
4. Tamarixetin	3, 5, 7, 3'	4'	S	2,530	8,910	n. d.
5. Ombuin	3, 5, 3'	7, 4'	S	1,070	3,360	n. d.

Table I. O-Methylating activity of cell-free extracts of citrus tissues with various flavonoid substrates [a].

[a] Using the standard assay described in the text.
[b] M, Merck; R, Karl Roth; S, synthesized by partial methylation [14] using diazomethane in dry pyridine.
[c] Total methylation expressed in cpm/mg protein.
n. d., not determined.

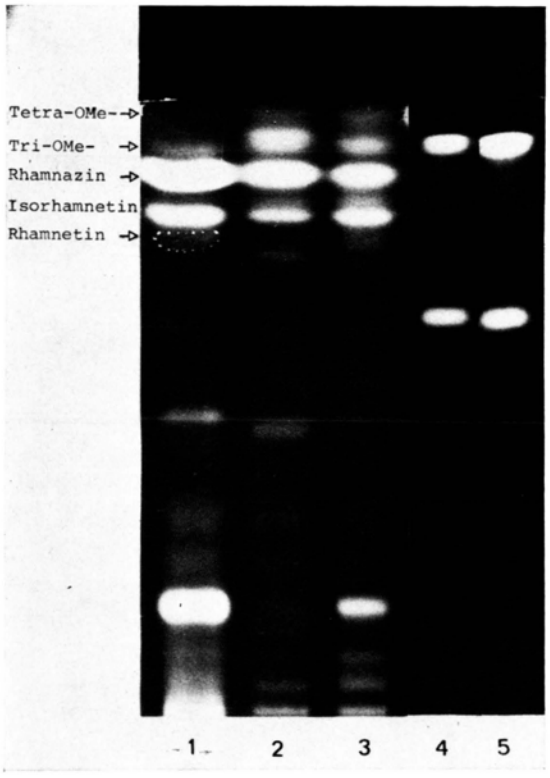

Fig. 1. Photograph of an autoradiograph of the O-methylated products of: 1. quercetin; 2. isorhamnetin; 3. rhamnetin; 4. tamarixetin; 5. ombuin, after chromatography in benzene-pyridine-formic acid (86:19:5). Each of the products was rechromatographed with reference compounds two-directionally (see text for details).

catalyzed the O-methylation of quercetin, though with different efficiencies. Each of the systems gave three products which were rigorously identified as rhamnetin, isorhamnetin and rhamnazin (Fig. 1). Their identity was confirmed by co-chromatography with reference compounds in a number of solvent systems. The fact that rhamnazin had always higher label incorporation than either rhamnetin or isorhamnetin (Fig. 1, columns 1 – 3) tends to suggest that the two latter compounds were intermendiate products of *para* and *meta* O-methylating enzymes of rings A and B, respectively. When either of these intermediates was used as substrate, it was further methylated to rhamnazin (Table I, Fig. 1) and possibly to a trimethyl ether derivative with a higher R_f value. Regardless of the source of crude enzyme preparation, rhamnetin was a better substrate than isorhamnetin (Table I, Fig. 1). Examination of the reaction products of both substrates indicated that they may have undergone demethylation [11] followed by remethylation. Due to the lack of sufficient amount of rhamnazin, we used its isomer (ombuin) as substrate. The latter was further methylated to a trimethyl ether derivative of quercetin (Fig. 1) whose identity was not further investigated. It is also interesting to note that tamarixetin was a good methyl acceptor, especially with the peel enzyme (Table I), though the exact position of methylation remains to be determined. Regardless of the position or degree of methylation of the substrate used, the efficiency of total methylation by cell-free extracts followed the order: callus tissue > peel > root, though the ratios of products may vary in the different tissue systems. This finding concurs with an earlier report [12] on the capacity of citrus callus tissue to synthesize a number of polymethylated flavones *in vitro*.

These results seem to indicate that citrus tissues contain at least two O-methyltransferases: one is a *meta*-directing enzyme that acts on ring-B and is possibly similar to those reported before [2 – 5], and the other, *para*-directing and acts on ring-A. These two enzymes may not necessarily be flavonoid-specific since caffeic acid and esculetin were methylated by citrus cell-free extracts to their respective *m*- and *p*-O-methyl derivatives (ferulic and isoferulic acids; scopoletin and isoscopoletin, respectively) in a manner similar to that of the yeast enzymes [13]. Further work is in progress to isolate and purify both enzymes. Furthermore, flavonoid compounds with specific ring-A substitution are being synthesized in order to study the pattern of their methylation *in vitro*.

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[1] J. B. Harborne, Comparative Biochemistry of the Flavonoids, Academic Press, New York 1967.
[2] J. Ebel, K. Hahlbrock, and H. Grisebach, Biochim. Biophys. Acta **268,** 313 (1972).
[3] H. Wengenmayer, J. Ebel, and H. Grisebach, Eur. J. Biochem. **50,** 136 (1974).
[4] J. E. Poulton, K. Hahlbrock, and H. Grisebach, Arch. Biochem. Biophys. **180,** 543 (1977).

[5] R. Sütfeld and R. Wiermann, Biochem. Physiol. Pflanzen **172,** 111 (1978).
[6] J. B. Harborne, Phytochemistry (L. P. Miller, ed.), **Vol. II,** pp. 344, Van Nostrand Reinhold 1973.
[7] H. E. Stafford, Ann. Rev. Plant Physiol. **25,** 459 (1974).
[8] K. Hahlbrock and H. Grisebach, The Flavonoids (J. B. Harborne, T. J. Mabry, and H. Mabry, eds.), **Vol. II,** pp. 866, Academic Press, New York 1975.
[9] H. Grisebach and K. Hahlbrock, Recent Advances in Phytochemistry (V. C. Ruenckles and E. E. Conn, eds.), **Vol. 8,** pp. 21, Academic Press, New York 1974.
[10] L. Hörhammer, H. Wagner, and K. Hein, J. Chromatogr. **13,** 235 (1964).
[11] W. Barz and W. Hösel, The Flavonoids (J. B. Harborne, T. J. Mabry, and H. Mabry, eds.), **Vol. II,** pp. 916, Academic Press, New York 1975.
[12] G. Brunet and R. K. Ibrahim, Z. Pflanzenphysiol **69,** 152 (1973).
[13] D. Müller-Enoch, H. Thomas, W. Streng, W. Wildfeuer, and O. Haferkamp, Z. Naturforsch. **31 c,** 509 (1976).
[14] H. M. Fales, T. M. Jaoni, and J. F. Babashak, Anal. Chem. **45,** 2302 (1973).