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Unusual pseudosubstrate specificity of a novel 3,5-dimethoxyphenol *O*-methyltransferase cloned from *Ruta graveolens* L.

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Abstract

A cDNA was cloned from *Ruta graveolens* cells encoding a novel *O*-methyltransferase (OMT) with high similarity to orcinol or chavicol/eugenol OMTs, but containing a serine-rich N-terminus and a 13 amino acid insertion between motifs IV and V. Expression in *Escherichia coli* revealed *S*-adenosyl-L-methionine-dependent OMT activity with methoxylated phenols only with an apparent K_m of 20.4 for the prime substrate 3,5-dimethoxyphenol. The enzyme forms a homodimer of 84 kDa, and the activity was insignificantly affected by 2.0 mM Ca^{2+} or Mg^{2+}, whereas Fe^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+} or Hg^{2+} were inhibitory (78–100%). Dithiothreitol (DTT) suppressed the OMT activity. This effect was examined further, and, in the presence of Zn^{2+} as a potential thiol methyltransferase (TMT) cofactor, the recombinant OMT methylated DTT to DTT-monomethylthioether. Sets of kinetic OMT experiments with 3,5-dimethoxyphenol at various Zn^{2+}/DTT concentrations revealed the competitive binding of DTT with an apparent K_i of 52.0 μM. Thus, the OMT exhibited TMT activity with almost equivalent affinity to the thiol pseudosubstrate which is structurally unrelated to methoxyphenols.

Keywords: *Ruta graveolens* L.; Trimethoxyphenol; 3,5-Dimethoxyphenol *O*-methyltransferase; Dithiothreitol *S*-methyltransferase; *O*-Methyltransferase pseudosubstrate

Numerous *S*-adenosyl-L-methionine-dependent *O*-methyltransferases (OMTs)[3] have been isolated from plants methylating nucleophilic hydroxyl or carboxyl groups of a variety of substrates [1–5]. Most of these enzymes show preferences for aromatic substrates with narrow specificity concerning the pattern of ring substitution [6–9], but multifunctional OMTs have also been reported [10,11]. Noteworthy in the latter instances, the OMTs accepted structurally related substrates only, i.e., various phenolics. Extensive studies were conducted on "lignin-specific" OMTs from various plants, and the assignment of two classes was proposed [12]: low molecular weight (23–27 kDa subunits), Mg^{2+}-dependent OMTs which do not accept caffeic acid as substrate were grouped to class I, and OMTs (38–43 kDa subunits) methylating caffeic acid or caffeyl aldehyde and caffeyl alcohol (COMTs) independently of Mg^{2+} were designated

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[3] *Abbreviations used:* SAM, *S*-adenosyl-L-methionine; DTT, 1,4-dithiothreitol; LC–MS, liquid chromatography-mass spectrometry; LSC, liquid scintillation counting; OMT, *O*-methyltransferase; SEC, size exclusion chromatography; TLC, thin-layer chromatography; TMT, thiol methyltransferase.; RLM-RACE, RNA-ligase mediated rapid amplification of cDNA ends; *Pmg*, elicitor from *Phytophthora sojae* (formerly *P. megasperma* f. sp. *glycinea*).

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class II. Class I enzymes were initially identified as caffeoyl-CoA OMTs [1] methylating the guaiacyl moiety in lignin biosynthesis [13], whereas class II COMTs were considered to methylate in situ primarily 5-hydroxyconiferaldehyde residues at a later stage in the lignin pathway, a conclusion that was supported recently by phenolic profiling of COMT-deficient transformants [14]. An N-terminal domain was proposed to determine the specificity of class I OMT [15]. However, the subclassification of "lignin-specific" OMTs on the basis of substrate specificities is not absolute, because an OMT accepting both caffeoyl-CoA and caffeic acid was reported from loblolly pine [16] and, more recently, a class I OMT with broader substrate specificity was reported from *Mesembryanthemum crystallinum* [17]. Furthermore, a class I OMT from *Ammi majus* was shown to switch substrate specificity towards caffeic and 5-hydroxyferulic acid when Mg^{2+} was replaced by Mn^{2+} or Co^{2+} in the assays [18].

Class II OMTs which are related to catechol OMT [19] appear to be even less discriminate in their choice of substrates. A number of the respective cDNAs was functionally expressed in *Escherichia coli*, and several of these recombinant OMTs were shown to methylate both phenylpropanoids and flavonoids [20,21] or benzaldehydes [22]. The numerous COMT polypeptide sequences deposited in data libraries firmly revealed the conserved motifs required for SAM-binding [2,12,23]. However, the molecular basis of substrate specificity still remains to be established, because residues determining the individual substrate affinities were assigned in only a few instances [4,24,25], and the predictive value of the alignments of class II OMT sequences is still under debate [21]. The search has recently been extended to related OMTs involved in the formation of volatile phenolics as constituents of floral fragrance, i.e., phenylpropenes [26–28] and methoxybenzenes [29,30]. While in case of the phenylpropene OMTs from basil the exchange of a single amino acid residue was shown to modulate the relative specificities of chavicol vs. eugenol OMTs [26], the residues controlling specificity in methoxybenzene OMTs (orcinol OMTs and phloroglucinol OMT) remain to be elucidated. However, phylogenetic analysis revealed that class II OMTs fall into two distinct categories: the first includes mainly COMTs, preferring caffeic acid as substrate, while the second category designates enzymes with a more diverse range of specificities [26,29]. Both chavicol and eugenol OMTs as well as orcinol and phloroglucinol OMTs belong to the second distinct category of OMTs [29]. The concerted action of the latter two enzymes is required to develop the full bouquet of rose fragrance, because 1,3,5-trimethoxybenzene as the major scent component besides 3,5-dimethoxytoluene is generated from phloroglucinol via 3,5-dihydroxyanisole and 3,5-dimethoxyphenol [29]. Orcinol OMT was shown in vitro to catalyze both the methylations converting 3,5-dihydroxyanisole to 1,3,5-trimethoxybenzene.

This study describes a novel methoxybenzene OMT from *Ruta graveolens* L., the common rue, which appears to be also involved in the formation of volatile 1,3,5-trimethoxybenzene but clearly differs from orcinol OMT. Moreover, the enzyme shows a puzzling thioltransferase activity towards the pseudosubstrate dithiothreitol (DTT) which has not been reported before.

Results

cDNA cloning and sequence alignments

Suspension cultures of *R. graveolens* were previously established for the investigation of acridone alkaloid biosynthesis [31,32], and the RNA isolated from cells that had been induced for 2–4 h with crude fungal elicitor were used to clone acridone synthase cDNA [33]. The biosynthesis of acridone alkaloids requires several methylations, i.e., the N-methylation of anthranilate, and the RNA was therefore employed also for RT-PCR at rather low stringency with degenerate oligonucleotide primers designed for the amplification of class II OMTs [6]. Amplicons of about 200 bp length were subcloned and sequenced for provisional verification of OMT-relationship. A PCR fragment of 214 bp was finally chosen for the design of gene specific primers, and the full-size cDNA of 1590 nucleotides was generated by RACE and RLM-RACE. DNA sequencing revealed an open reading frame of 1122 bp encoding a 374 residue polypeptide of M_r 41,559.

Comparison of the translated polypeptide with eight class II OMTs from data base accessions (Fig. 1) using multiple CLUSTAL-W [34] alignments revealed 61–62% sequence similarity with chavicol and orcinol OMTs at a 46–50% level of identity. A little less identity was noticed with a flavonoid OMT from *Catharanthus roseus* (41%) and reticuline OMT from *Papaver somniferum* (36%), whereas the identity with caffeic acid OMTs was in the range of 30% only. The five sequence elements conserved in SAM-dependent OMTs and proposed to be involved in SAM and metal binding, mainly inferred from X-ray diffraction analysis of crystallyzed rat liver catechol or alfalfa chalcone and isoflavone OMTs [19,25], were also recognized in the *Ruta* polypeptide (Fig. 1, regions I–V). These elements are ubiquitously present in plant class II OMTs [2,21], and the sequence relationship of the *Ruta* enzyme was further corroborated by comparison with 50 methyltransferases annotated in databases using the program WU-Blast2 (EMBL, Heidelberg), which did not reveal any significant sequence similarity to plant class I OMTs or *S*- and *N*-methyltransferases. Thus, the cDNA isolated from *R. graveolens* was provisionally assigned as RgOMT (GenBank Accession No. AY894417). Nevertheless, two peculiar features