Purification and properties of a new S-adenosyl-L-methionine:flavonoid 4'-O-methyltransferase from carnation (Dianthus caryophillus L.)

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Purification and properties of a new *S*-adenosyl-L-methionine:flavonoid 4′-*O*-methyltransferase from carnation (*Dianthus caryophyllus* L.)

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A new enzyme, *S*-adenosyl-L-methionine:flavonoid 4′-*O*-methyltransferase (EC 2.1.1.-) (F 4′-OMT), has been purified 1 399-fold from the tissues of carnation (*Dianthus caryophyllus* L). The enzyme, with a molecular mass of 43–45 kDa and a pI of 4.15, specifically methylates the hydroxy substituent in 4′-position of the flavones, flavanones and isoflavones in the presence of *S*-adenosyl-L-methionine. A high affinity for the flavone kaempferol was observed ($K_m = 1.7$ μM; $V_{max} = 95.2$ μmol·min^{-1}·mg^{-1}), while other 4′-hydroxylated flavonoids proved likewise to be suitable substrates. Enzyme activity had no apparent Mg^{++} requirement but was inhibited by SH-group reagents. The optimum pH value for F 4′-OMT activity was found to be around neutrality. Kinetic analysis of the enzyme bi-substrate reaction indicates a Ping-Pong mechanism and excludes the formation of a ternary complex. The F 4′-OMT activity was increased, in both *in vitro* and *in vivo* carnation tissues, by the inoculation with *Fusarium oxysporum* f. sp. *dianthi*. The enzyme did not display activity towards hydroxycinnamic acid derivatives, some of which are involved, as methylated monolignols, in lignin biosynthesis; the role of this enzyme could be therefore mainly defensive, rather than structural, although its precise function still needs to be ascertained.

Keywords: *S*-adenosyl-L-methionine:flavonoid 4′-*O*-methyltransferase; *O*-methyltransferase; *Fusarium oxysporum* f. sp. *dianthi*; *Dianthus caryophyllus*; carnation.

O-Methyltransferases (OMTs) are important plant enzymes that are involved in several biochemical processes such as lignin biosynthesis [1] and methylation of various secondary metabolites [2]. In many cases, these enzymes may be associated to plant defense systems against pathogens and those OMTs belonging to the OMT II and OMT III classes have been recognized as pathogenesis-related enzymes, as they are inducible by an infection, and methylate efficaciously a broad spectrum of phenols associated to plant defensive processes [3]. As far as we know, OMT activity in carnation (*Dianthus caryophyllus* L) has not been investigated thoroughly yet. Reinhard and Matern [4] found in carnation an OMT activity related to the tissue defensive response towards *Phytophthora megasperma*. This enzymatic activity plays a fundamental role in the biosynthesis of methylated dianthramide-derivatives, the carnation phytoalexins. However, no data are available regarding the role of this enzymatic activity in the biosynthesis of methylated phenols other than the dianthramide-derivatives. In this respect, the object of the present investigation, the carnation cultivar 'Novada', known as one of the most resistant to *Fusarium oxysporum* f. sp. *dianthi* (*Fod*) [5,6], contains a constitutive methoxylated flavone, kaempferide (3,5,7-trihydroxy-4′-methoxyflavone) triglycoside, which displays an inhibitory activity towards the pathogen and is therefore involved in plant defense against the parasite [7]. Preliminary investigations on the artificially *Fod*-inoculated 'Novada' cultivar (P. Curir, unpublished results) evidenced the presence of an elicitable, specific *S*-adenosyl-L-methionine:flavonoid 4′-*O*-methyltransferase (EC 2.1.1.-) (F 4′-OMT) in plant tissues; this enzymatic activity proved able to convert kaempferol into kaempferide, which is the aglycone of the above mentioned antifungal constitutive kaempferide triglycoside, suggesting a possible involvement of this enzyme in plant defense. This prompted us to perform the present research, where we report the purification and characterization of F 4′-OMT from carnation. The hypothesis that this enzyme may have a role in carnation defensive processes against *Fod* infection is likewise discussed.

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Abbreviations: F4′-OMT, *S*-adenosyl-L-methionine:flavonoid 4′-*O*-methyltransferase; OMTs, *O*-methyltransferases; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine.
Enzyme: *S*-adenosyl-L-methionine:flavonoid 4′-*O*-methyltransferase (EC 2.1.1.-).

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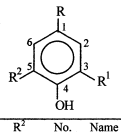
Materials and methods

Chemicals

S-adenosyl-L-methionine (AdoMet) and *S*-adenosyl-L-homocysteine (AdoHcy) were obtained from Sigma-Aldrich. 4-Hydroxybenzoic acid (I), gallic acid (3,4,5-tri-



No.	Name	R	R^1	R^2	No.	Name	R	R^1	R^2
I	4-Hydroxybenzoic acid	-COOH	-H	-H	III	*p*-Coumaric acid	-CH=CHCOOH	-H	-H
II	Gallic acid	-COOH	-OH	-OH	IV	Caffeic acid	-CH=CHCOOH	-OH	-H

Fig. 1. Molecular structures of the hydroxybenzoic acid (I, II) and hydroxycinnamic acid (III, IV) derivatives assayed as substrates for the flavonoid 4′-OMT.

hydroxybenzoic acid) (II), *p*-coumaric acid (4-hydroxycinnamic acid) (III) and caffeic acid (3,4-dihydroxycinnamic acid) (IV) were purchased from Merck, (Fig. 1). Kaempferol (3,4′,5,7-tetrahydroxyflavone) (V), quercetin (3,3′,4′,5,7-pentahydroxyflavone) (VII), rutin (quercetin-3-O-rutinoside) (VIII), datiscetin (2′,3,5,7-tetrahydroxyflavone) (IX), apigenin (4′,5,7-trihydroxyflavone) (X), luteolin (3′,4′,5,7-tetrahydroxyflavone) (XI), isorhamnetin (3,4′,5,7–tetrahydroxy-3′-methoxyflavone) (XII), kaempferide (3,5,7–trihydroxy-4′-methoxyflavone) (XIII) (Fig. 2), 4′-hydroxyflavanone (XVI), eriodictyol (3′,4′,5,7-tetrahydroxyflavanone) (XVII) (Fig. 3), genistein (4′,5,7-trihydroxyisoflavone) (XIX), 3′,4′,7-trihydroxyisoflavone (XX), and biochanin A (5,7–dihydroxy–4′-methoxyisoflavone) (XXI) (Fig. 4), were purchased from Extrasynthèse, Lyon, France. Before use, all the compounds were purified using column chromatography according to Curir *et al.* [8]. The flavone triglycoside, kaempferol 3-*O*-β-D-glucopyranosyl-[1 → 4]-*O*-α-L-rhamnopyranosyl-[1(r)2]-β-D-glucopyranoside (VI) (Fig. 2) was extracted and purified from *Allium neapolitanum* Cyr. according to Carotenuto *et al.* [9]. Caffeoyl (3,4-dihydroxycinnamoyl) CoA was prepared following the protocol of Stöckigt and Zenk [10], identified and quantified spectrophotometrically according to Lüderitz *et al.* [11].

Buffer systems

The following buffer solutions were used: Buffer A, 25 mM Tris/HCl, pH 7.0; Buffer B, 0.1 M NaP_i, pH 7.0; Buffer C, 20 mM Bis/Tris/Propane {BTP; 1,3-bis[tris(hydroxymethyl)-methylamino]propane}, pH 7.0.

In vivo plant material

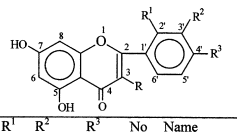
The carnation cultivar 'Novada' was obtained from the DLO Institute, Wageningen, Holland. Two hundred rooted cuttings were planted in 250-mm diameter pots, on steam-sterilized soil, and grown for 8 months under greenhouse conditions with a natural photoperiod.

In vitro plant material

Stem internodal explants, 10 mm tall, from *in vivo* 'Novada' plants were surface sterilized with a NaOCl solution, 0.8% free chlorine, for 10 min and further rinsed three times with sterile double distilled water. Explants were then transferred into test tubes (25 × 150 mm, Kaputs, BellCo, USA) containing Murashige and Skoog macro- and micro-elements, iron chelates and vitamins [12], plus 50 $mg \cdot L^{-1}$ ascorbic acid, 30 $g \cdot L^{-1}$ sucrose, 5 $\mu mol \cdot L^{-1}$ 2,4-dichlorophenoxyacetic acid, 2 $\mu mol \cdot L^{-1}$ 3-indolylacetic acid (IAA), 0.2 $\mu mol \cdot L^{-1}$ benzylaminopurine, 8.0 $g \cdot L^{-1}$ Difco Bacto agar, pH 5.8 prior to autoclaving. Media were sterilized for 15 min at 121 °C and 1 atm pressure. Explant growth conditions were: 22 °C temperature, 12 h photoperiod, with an illumination of 180 $\mu E \cdot m^{-2} \cdot s^{-1}$. After 1 month of culture, the friable callus developed from the starting explants was transferred onto fresh medium and subcultured for 2 months under the same conditions. Fresh callus (3 g) were then transferred into a 100-mm diameter Petri dish, filled with 8 mL of the above mentioned culture medium: a total of 400 dishes were prepared and used in the further steps of the experiments.

Fungal material

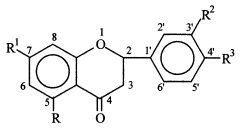
Fod pathotype 2 was used in the experiments, as the most widespread and pathogenic race among those infecting carnation throughout the world [5]. P 75 strain inocula were obtained from A. Garibaldi (University of Torino, Italy) who also determined species and pathotype. Mycelial explants were inoculated into 1 L flasks, containing Czapek broth, kept in agitated culture (80 strokes per min) for 12 days to induce conidia formation.



No	Name	R	R^1	R^2	R^3	No	Name	R	R^1	R^2	R^3
V	Kaempferol	-OH	-H	-H	-OH	XIII	Kaempferide	-OH	-H	-H	$-OCH_3$
VI	Kaempferol triglycoside	*	-H	-H	-OH	XIV	Kaempferide triglycoside	*	-H	-H	$-OCH_3$
VII	Quercetin	-OH	-H	-OH	-OH						
VIII	Rutin	-*O*-rutinosyl	-H	-OH	-OH						
IX	Datiscetin	-OH	-H	-OH	-OH						
X	Apigenin	-H	-H	-H	-OH	XV	Acacetin	-H	-H	-H	$-OCH_3$
XI	Luteolin	-H	-H	-OH	-OH						
XII	Isorhamnetin	-OH	-H	$-OCH_3$	-OH						

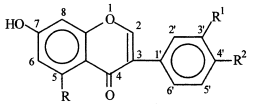
* -*O*-β-D-glc-(1→4)-*O*-α-L-rha-(1→2)-β-D-glc

Fig. 2. Molecular structures of the flavones (V-XII) assayed as substrates to the flavonoid 4′-OMT and the transformation products (XIII-XV).



No	Name	R	R^1	R^2	R^3	No	Name	R	R^1	R^2	R^3
XVI	4'-Hydroxyflavanone	-H	-H	-OH	-H	XVIII	4'-Methoxyflavanone	-H	-H	-H	$-OCH_3$
XVII	Eriodictyol	-OH	-OH	-OH	-OH						

Fig. 3. Molecular structures of the flavanones (XVI-XVII) assayed as substrates to the flavonoid 4′-OMT and the transformation product (XVIII).



No	Name	R	R^1	R^2	No	Name	R	R^1	R^2
XIX	Genistein	-OH	-H	-OH	XXI	Biochanin A	-OH	-H	$-OCH_3$
XX	3',4',7-Trihydroxyisoflavone	-H	-OH	-OH					

Fig. 4. Molecular structures of the isoflavones (XIX-XX) assayed as substrates to the flavonoid 4′-OMT and the transformation product (XXI).

In vivo and *in vitro* inoculation of plant material

One hundred fully developed *in vivo* carnation plants were individually stem-inoculated (10 branches, 200 mm long, for each plant), according to the method of Baayen and Elgersma [13], with a 500-μL drop of *Fod* conidial suspension at a concentration of 9×10^6 conidia·mL^{-1}; 20 additional plants, inoculated with a 500-μL drop of double distilled water, represented the control. Stem parts 20–25 mm above and below the inoculation site were collected 24, 48 and 72 h later, respectively, and used in the further analyses.

Among the 400 *in vitro* carnation calluses set in Petri dishes as described previously, 250 actively growing ones were selected and surface-inoculated individually with a 100-μL drop of the same conidial suspension used for the *in vivo* material; a further 70 calluses were inoculated with a 100-μL drop of double distilled water and represented the control. After, respectively, 24, 48 and 72 h of culture under the growth conditions already specified for the *in vitro* material, the calluses were collected and used in the following steps.

Measurement of the F 4′-OMT activity

Standard assay conditions. The F 4′-OMT activity was assayed through a modified protocol described previously [14]. Enzyme solution (2 mL of up to 8 μM) was incubated with 1 mL buffer B containing 50 μmol·L^{-1} AdoMet and 100 μmol·L^{-1} kaempferol (V). After 25 min incubation at 25 °C, the reaction was stopped by the addition of two drops 10 M HCl. Product formation was determined analyzing the reaction mixture through HPLC, measuring the nmols of kaempferide (XIII) formed per min per mg protein and expressing the activity as nkat mg^{-1}·protein. Controls with no enzyme or no AdoMet were included. When the enzyme crude activity within plant tissues was investigated, analyses were performed on the same amounts of both *Fod*-inoculated and *Fod*-uninoculated tissues, with the aim of assessing if the F 4′-OMT activity could be associated, to some extent, to the tissue's defense response.

HPLC analyses. HPLC analyses were carried out using a Merck-Hitachi Chromatograph (mod. L-6200), equipped with a diode array detector (mod. L-6200) set at 350 nm wavelength for flavonoids, and 280 nm for simple phenols, respectively. An Ultracarb ODS-30 column was used, 150 × 4.6 mm, 5 μm particle size (Phenomenex, Torrance, USA), thermostated at 25 °C. The solvent was a mixture of 0.05 M NaP_i buffer, pH 3, and acetonitrile (6 : 1, v/v); separation was performed isocratically, at a flow rate of 1 mL·min^{-1}, and the volume of injected samples was 10 μL. The amounts of the residual initial phenolic substrate and the transformation product, derived from incubation with enzyme preparations, were determined in samples by comparing their peak-integrated areas with those obtained from known concentrations of the respective standards.

Kinetic analysis and studies with different substrates. Kinetic analyses were performed following Jencks [15] and Nelson & Cox [16]. Kinetic analyses were carried out at neutral pH in buffer B, using 0.8 μg purified F 4′-OMT per assay, at AdoMet concentrations from 100 to 300 μM and 100 μM of each phenol to be tested, purified through column chromatography according to a former procedure [8]; the flavone VI (kaempferol triglycoside) was purified according to Carotenuto *et al.* [9]. After 25 min incubation at 25 °C, the reaction was stopped by the addition of two drops of 10 M HCl.

Aliquots of each reaction mixture were analyzed through column chromatography [7] to separate and purify both the assayed substrates and the respective possible methylated compound; the reaction mixture containing kaempferol triglycoside (VI) as a substrate was chromatographed by MPLC on silica gel RP-18 using a linear gradient elution profile from H_2O 100% to MeOH 100% in order to purify the possible related kaempferide derivative. The components of each reaction mixture, after their chromatographic separation and purification, were submitted to ^{1}H NMR (nuclear magnetic resonance) and FABMS (fast atom bombardment mass spectrum) analyses to ascertain the respective molecular structure, as described below. Once the component identity was determined, every reaction solution was analyzed using HPLC (as above), which proved to be a

reliable analytical tool to assay OMT activities [14,17]. The concentration of both the assayed compound and its respective transformation product was determined by comparison of peak data with those obtained from authentic standards chromatographed at different known concentrations. The specific F 4′-OMT activity towards a substrate was measured as nmols of methylated compound formed from its corresponding unmethylated precursor per min per mg protein and expressed as nkat per mg protein. Kinetic values (V_{max} and K_m) were determined with the Lineweaver–Burk plot method at a saturating concentration of AdoMet. V_{max} is expressed in $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ and K_m in μM. Assays to calculate kinetic values were repeated 3 times.

1H NMR spectrometry and FABMS analyses. ^1H NMR spectra were recorded at 500 MHz on a Bruker AMX-500 spectrometer in CD_3OD. Chemical shifts were referred to the residual solvent signal (CD_3OD: δ 3.34). FABMS in negative ion mode were recorded in a glycerol matrix on a VG Prospec (Fisons Instruments, Danvers, NJ, USA) instrument (Cs^+ ions of energy of 4 kV).

Extraction and purification of F 4′-OMT

All the purification steps were carried out at 4 °C temperature. The enzyme was concentrated at various steps of purification using collodion bags with 5 kDa cut-off (Sartorius, Gottingen, Germany). The chromatography eluates were monitored at 280 nm for proteins by a Bio-Rad econo-UV-monitor (Bio-Rad, Richmond, USA).

Extraction and $(NH_4)_2SO_4$ fractioning. *Fod*-inoculated and uninoculated *in vitro* calluses and *in vivo* stem segments were utilized. For each different type of material, 200 g fresh tissues at a time were homogenized in 2 L $(CH_3)_2CO$ containing 3% MeOH, by means of a Blendmaster blender (Proctor-Silex, Washington, USA). Each homogenate was centrifuged at 5000 ***g*** for 30 min, and the supernatant discarded; the sediment was re-suspended in the extraction solution and collected by centrifugation: this step was repeated until the supernatant appeared as a clear solution. Each sediment was then vacuum-dried and extracted overnight with 200 mL buffer A shaken by a magnetic stirrer; the obtained solutions were filtered through cheesecloth, centrifuged as above and the collected surnatant was concentrated to 50 mL to originate the respective 'protein crude extract'. A first protein fractionation was obtained adding $(NH_4)_2SO_4$ to the various crude solutions, to reach three different saturation percentages of: 40, 60 and 90; the corresponding protein precipitates were collected by centrifugation, redissolved in and dialyzed against buffer A, concentrated as above and tested for their F 4′-OMT activity. The enzymatically active fractions were then submitted to the further purification phases.

DEAE-Cellulose chromatography. Aliquots (1–3 mL) of each protein extract from $(NH_4)_2SO_4$ fractionation were loaded, at various times, onto a chromatography column (400 × 20 mm) filled with DEAE-Cellulose (diethylaminoethyl-cellulose) (Whatman) packed and equilibrated with the buffer A; the elution was performed with 200 mL of a 0–0.5 M linear gradient of NaCl in buffer A, at a flow rate of $0.5\ mL \cdot min^{-1}$. The obtained 3 mL fractions were assayed for their F 4′-OMT activity and those proved active were pooled and desalted through dialysis, overnight, against buffer A. The obtained enzyme-containing fraction was concentrated to 2 mL as above.

DEAE-Sepharose chromatography. Samples (2 mL) were loaded onto a DEAE-Sepharose (diethylaminoethyl-sepharose) column (250 × 20 mm) packed with buffer B and eluted with 80 mL of a 0–0.3 M linear gradient of NaCl in buffer B, at a flow rate of $0.4\ mL \cdot min^{-1}$. Fractions containing an F 4′-OMT activity were pooled, dialyzed and concentrated as above to 2 mL.

Gel-filtration chromatography on Sephacryl S-110. The concentrated samples were loaded onto a Hi-Prep Sephacryl S-100 HR prepacked column, 16 × 600 mm (Pharmacia), packed with buffer C; the elution was performed with 200 mL of a 0–0.15 M linear gradient of NaCl in buffer C, at a flow rate of $1\ mL \cdot min^{-1}$, collecting 2 mL fractions. The F 4′-OMT-containing fractions were pooled, desalted through dialysis and the obtained solution was concentrated to 1 mL as already described.

Ion-exchange chromatography on Q-Sepharose. The 1 mL samples were applied to a Hi-Trap Q Sepharose XL 5 × 1 mL (anion exchanger), prepacked column (Pharmacia); the elution was performed using 70 mL of a 0–0.3 M linear gradient profile of NaCl in buffer B, at a flow rate of $0.8\ mL \cdot min^{-1}$, collecting 1 mL fractions. The fractions with an F 4′-OMT activity were pooled, desalted overnight through dialysis against the buffer B and concentrated as described previously: these fractions were considered as pure enzyme preparations.

Protein quantitation

Total protein concentration was measured at every step according to Lowry *et al.* [18], using a suitable calibration curve obtained with BSA.

Molecular mass determination

Molecular mass of the pure F 4′-OMT enzyme was first calculated by gel filtration, using a Superdex 200 (Amersham) prepacked column (3.2 × 300 mm), calibrated with RNAase A (molecular mass 13.7 kDa), chymotrypsinogen A (25.0 kDa), ovalbumin (45.0 kDa), BSA (67.0 kDa), and Blue Dextran 2,000, the latter used to determine the column void. The column had been equilibrated with buffer A containing 200 mM NaCl, and was eluted with the same solvent at a flow rate of $0.5\ mL \cdot min^{-1}$. The enzyme molecular mass was then re-checked through flatbed PAGE, using a PhastSystem™ (Amersham) electrophoresis system and precast high density PhastGel slabs (43 × 50 × 0.45 mm). Runs were performed at 500 V, 10 mA, 5 W, 8 °C. The markers used were: phosphorylase B (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.4 kDa).

pI determination

The pI of purified F 4′-OMT was determined through PAGE isoelectrofocusing (IEF), using the PhastSystem electrophoresis apparatus (as above) and precast PhastGel minislabs, containing carrier ampholytes ensuring a pH range from 3.0 to 9.0, checked by a pHmeter (Orion Research, Beverly, MA, USA) equipped with a flat-point, surface electrode. Runs were performed at 300 V, 18 mA, 15 W, 8 °C, using as reference markers: pepsinogen (pI 2.80), amyloglucosidase (pI 3.50), methyl red (pI 3.75), glucose oxidase (pI 4.15), trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), carbonic anhydrase B (pI 5.85). Gels were stained with the PhastGel protein silver staining kit (Amersham, Uppsala, Sweden).

Results

Purification of F 4′-OMT

The whole sequence of chromatographic steps needed to be accomplished as rapidly as possible, as the enzyme proved to quickly loose its activity in the course of time: a storage period of 2 weeks at −20 °C caused a ≈ 50% loss of activity.

The different phases of F 4′-OMT purification are presented in Table 1. The enzyme was purified 1399-fold, to obtain a final specific activity of 1175 nkat·mg protein^{-1}. From crude total protein extracts, the F 4′-OMT activity was first obtained through precipitation with $(NH_4)_2SO_4$ 60% saturation. The first two chromatography steps were particularly useful in removing 9/10 of the contaminant proteins. The further gel-filtration and ion exchange chromatographies allowed the enzyme's final purification. In particular, when the Hi-Prep 16/60 Sephacryl S-100 HR matrix was used, all the F 4′-OMT activity was recovered from the fractions 44–63 (Fig. 5); with Hi-Trap Q-Sepharose XL chromatography the pure enzyme was eluted in the fractions 38–42 (Fig. 6). At the end of the latter purification phase, PAGE runs were performed in order to check the degree of enzyme purity; electrophoresis evidenced a single enzymatic band and no other contaminant protein was detectable (Fig. 7). This enzyme band proved to contain a single protein that did not split into subunits when subjected to the SDS treatment: further PAGE runs, carried out under denaturing conditions, confirmed that it consists actually of a unique enzymatic protein.

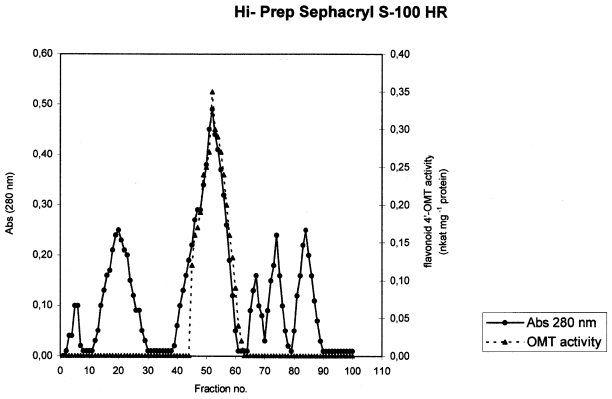


Fig. 5. Purification of the flavonoid 4′-OMT through gel filtration. Enzymatic activity is expressed as nkat·mg protein^{-1}.

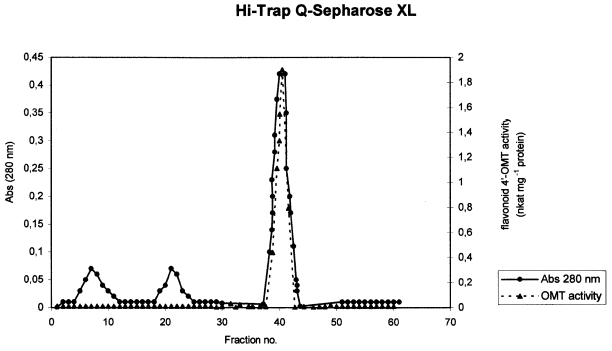


Fig. 6. Purification of the flavonoid 4′-OMT through ion exchange chromatography. Enzymatic activity is expressed as nkat.mg protein^{-1}.

Molecular mass and pI determination of F 4′-OMT. The molecular mass of the pure enzyme was calculated both through gel-filtration and PAGE (Fig. 7) in the presence of suitable protein markers, and was determined to be 43–45 kDa. This value is related to the whole enzyme that does not consist of subunits, as mentioned earlier. The enzyme pI, evaluated by means of IEF, is around 4.15: in fact, the purified F 4′-OMT band, electrophoresed under pH gradient conditions, stops at the migration level of the glucose oxidase marker band, having just the above pI value.

Table 1. Purification steps of s-adenosyl-methionine:flavonoid 4′-*O*-methyltransferase from carnation (*Dianthus caryophyllus*) stem. DEAE-Seph, diethylaminoethylcellulose-sepharose; Hi-Prep Seph, Hi-Prep 16/60 sephacryl S-100 high resolution (gel filtration); Hi-Trap Q-Seph, Hi-Trap Q Sepharose XL 5 × 1 mL (anion exchanger).

Purification step	Total activity (nkat)	Total protein (mg)	Specific activity (nkat·mg^{-1})	Purification (*n*-fold)	Recovery of activity %
Crude extract	293.5	350	0.84	–	100
$(NH_4)_2SO_4$ (60%)	243	180	1.35	1.61	83
DEAE	197.2	85	2.32	2.76	67.2
DEAE-Seph	171	18	9.5	11.3	58
Hi-Prep Seph	153.2	1	153.2	182	52
Hi-Trap Q-Seph	23.5	0.02	1175	1399	8

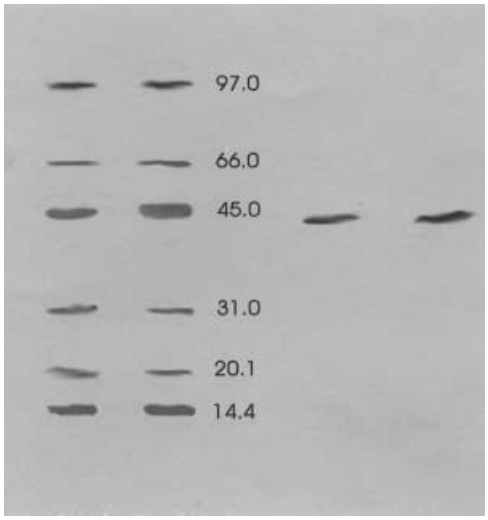


Fig. 7. PAGE of the purified flavonoid 4′-OMT from carnation. The position of molecular mass markers are indicated in kDa.

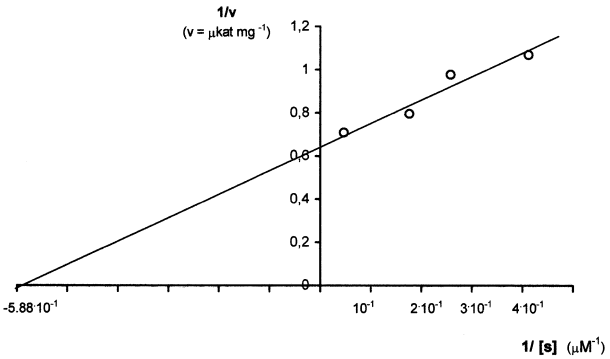


Fig. 8. K_m determination of the flavonoid 4′-OMT towards kaempferol (V) (K_m = 1.7 μM) through the Lineweaver–Burk plot of 1/*v* vs. 1/[s]. Enzyme concentration was 2 μM while the substrate was used at concentrations ranging from 0.15 to 6 μM.

F 4′-OMT activity towards the different assayed substrates, transformation products and kinetic analysis. The enzyme became inactive when the assayed substrates were the hydroxybenzoic acids: 4-hydroxybenzoic acid (I) and 3,4,5-trihydroxybenzoic acid (gallic acid) (II), or the hydroxycinnamic acids: 4-hydroxycinnamic acid (*p*-coumaric acid) (III) and 3,4-dihydroxycinnamic acid (caffeic acid) (IV) (Fig. 1); the enzyme was likewise inactive when caffeoyl-CoA was assayed as a possible methyl acceptor. The enzyme displayed its activity towards the hydroxy group in the 4′-position of some flavones, flavanones, and isoflavones. Kaempferol (V), kaempferol triglycoside (VI), apigenin (X), 4′-hydroxyflavanone (XVI) and genistein (XIX) behaved as suitable substrates for the enzyme, and gave the corresponding 4′-methoxy compounds (Figs 2,3,4); the identity of these was determined through ^{1}H NMR and FABMS analyses.

3,5,7-trihydroxy-4′-methoxyflavone (kaempferide, XIII). ^{1}H NMR (CD_3OD): δ 6.19 (1H, d, J = 1.6 Hz, H-6), 6.41 (1H, d, J = 1.6 Hz, H-8), 8.18 (2H, d, J = 8.5 Hz, H-2′ and H-6′), 7.05 (2H, d, J = 8.5 Hz, H-3′ and H-5′), 3.88 (3H, s, OCH_3). FABMS *m*/*z* 299 $(M-H)^-$.

3-*O*-β-D-glucopyranosyl-[1(r)4]-*O*-α-L-rhamnopyranosyl-[1 → 2]-β-D-glucopyranoside (kaempferide triglycoside, XIV). ^{1}H NMR (CD_3OD): δ 5.69 (1H, d, J = 7.5 Hz, H-1 inner glc), 5.20 (1H, bs, H-1 rha), 4.50 (1H, d, J = 7.8 Hz, H-1 external glc), 6.24 (1H, d, J = 1.8 Hz, H-6), 6.42 (1H, d, J = 1.8 Hz, H-8), 8.02 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 6.98 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 3.41 (3H, s, OCH_3). FABMS *m*/*z* 769 $(M-H)^-$.

5,7-dihydroxy-4′-methoxyflavone (acacetin, XV). ^{1}H NMR (CD_3OD): δ 6.62 (1H, s, H-3), 6.19 (1H, d, J = 2.0 Hz, H-6), 6.44 (1H, d, J = 2.0 Hz, H-8), 7.92 (2H, d, J = 8.5 Hz, H-2′ and H-6′), 7.18 (2H, d, J = 8.5 Hz, H-3′ and H-5′), 3.89 (1H, s, OCH_3). FABMS *m*/*z* 283 $(M-H)^-$.

4′-methoxyflavanone (XVIII). ^{1}H NMR (CD_3OD): δ 7.85 (1H, d, J = 8.5 Hz, H-5), 7.03 (1H, t, J = 8.5 Hz, H-6), 7.52 (1H, t, J = 8.5 Hz, H-7), 7.02 (1H, d, J = 8.5 Hz, H-8). 5.42 (1H, dd, J = 12.5 and 2.8, H-2), 3.14 (1H, dd, J = 17.0 and 12.5, Hax-3), 2.79 (1H, dd, J = 17.0 and 2.8, Heq-3), 7.43 (2H, d, J = 8.4 Hz, H-2′ and H-6′), 6.96 (2H, d, J = 8.4 Hz, H-3′ and H-5′); 3.80 (3H, s, OCH_3). FABMS *m*/*z* 253 $(M-H)^-$.

Biochanin A (XXI). ^{1}H NMR (CD_3OD): δ 6.20 (1H, d, J = 1.6 Hz, H-6), 6.32 (1H, d, J = 1.6 Hz, H-8), 7.45 (2H, d, J = 8.3 Hz, H-2′ and H-6′), 6.97 (2H, d, J = 8.3 Hz, H-3′ and H-5′), 8.07 (1H, s, H-2), 3.80 (3H, s, OCH_3). FABMS *m*/*z* 283 $(M-H)^-$.

On the contrary, quercetin (VII), rutin (VIII), luteolin (XI), eriodictyol (XVII), 3′,4′,7′-trihydroxyisoflavone (XX) bearing the hydroxy groups in 3′ and 4′-positions, and datiscetin (IX) bearing the hydroxy group in 2′ position were unaffected by the enzymatic activity (Figs 2,3,4). This shows that the hydroxy substituent must be placed in 4′-position and must not have an adjacent substituent, as isorhamnetin (XII) (Fig. 2). A high enzymatic affinity towards kaempferol (V) could be observed (K_m = 1.7 μM) (Fig. 8), with a calculated V_{max} of 95.2 μmol·min^{-1}·mg^{-1}; its glycosylated form, kaempferol triglycoside (VI), was likewise methylated, but the corresponding K_m could not be determined, due to the low availability of this substrate. The V_{max} and K_m with different substrates are shown in Table 2, together with the V_{max}/K_m ratio that reflects the enzyme catalytic efficiency. With respect to the tested flavones, among those without a hydroxy substituent in 3′-position only apigenin (X) was methylated by the enzyme that showed a high affinity for this substrate (K_m = 3.3 μM) but a halved V_{max} in comparison to kaempferol. Between the two assayed flavanones, 4′-hydroxyflavanone (XVI) proved to be a good substrate for the enzyme (K_m = 11.0 μM), with a V_{max} of 31.6 μmol·min^{-1}·mg^{-1}; eriodictyol (XVII) did not. Finally, the structure of the isoflavones does not prevent the enzyme's methylating activity, provided that the 4′-hydroxy substituent is maintained and that a hydroxy substituent in 3′-position is lacking. The enzyme affinity for the substrate is lower for the isoflavones: the enzyme K_m for genistein (XIX) is 73.5 μM, while V_{max} decreases to 3.8 μmol·min^{-1}·mg^{-1}. Enzyme affinity towards flavonoid substrates can therefore be summarized as follows: 4′-hydroxyflavones > 4′-hydroxyflavanones > 4′-hydroxyisoflavones; this rank holds true also when the catalytic efficiency (V_{max}/K_m) is considered (Table 2). Figure 9 shows the double-reciprocal plot of inhibition kinetic of AdoHcy. The obtained experimental data at increasing inhibitor

Table 2. Kinetic parameters of *S*-adenosyl-L-methionine:flavonoid 4′-*O*-methyltransferase versus different substrates and related transformation products. Each value represents the mean ± SD of five independent measurements. ND, not determined.

Substrate no.		Transformation product		Kinetic parameters		
Number	Name	Number	Name	V_{max} (μmol·min^{-1}·mg^{-1})	K_m (μM)	V_{max}/K_m
V	Kaempferol	XIII	Kaempferide	95.2 ± 0.31	1.7 ± 0.09	56.0 ± 0.19
VI	Kaempferol triglycoside	XIV	Kaempferide triglycoside	ND	ND	ND
X	Apigenin	XV	Acacetin	44.3 ± 0.6	3.3 ± 0.11	13.4 ± 0.5
XVI	4′-Hydroxyflavanone	XVIII	4′-Methoxyflavanone	31.6 ± 0.8	11.0 ± 0.39	2.87 ± 0.18
XIX	Genistein	XXI	Biochanin A	3.8 ± 0.1	73.5 ± 1.03	0.05 ± 0.01

concentrations [I] give raise to a family of lines with a common intercept on the $1/v$ axis but with different slopes. This indicates that V_{max} does not change in the presence of the inhibitor, regardless of its concentration, and that, therefore, the AdoHcy inhibition is competitive. Accordingly, the Michaelis–Menten equation:

$$V = V_{max}[S]/K_m + [S] \text{ becomes,}$$
$$V = V_{max}[S]/\alpha K_m + [S]$$

where,

$$\alpha = 1 + [I]/K_I \text{ and } K_I = [E][I]/[EI]$$

and [E] is enzyme concentration, [S] is substrate concentration. From the latter equation, K_I for AdoHcy was calculated as 12 ± 1 μM.

The analysis of the mechanisms for enzyme-catalyzed bi-substrate reaction was performed through double reciprocal plots of 1/v (1 μmol·min^{-1}) vs. different fixed kaempferol concentrations in the presence of four increasing AdoMet concentrations, 50, 65, 80 and 100 μM. From this analysis, a separate line is generated for each AdoMet concentration, which intersects the horizontal axis ($1/v$): all the obtained lines are parallel, indicating a ping-pong or double displacement mechanism, where no ternary complex is formed (Fig. 10). To support this hypothesis, when different concentrations of purified F 4′-OMT (0.1–0.4 μM) were assayed in the presence of AdoMet alone, without a methyl acceptor, AdoHcy accumulated in various amounts in the reaction solution, as evidenced through HPLC analyses (unpublished data). This would demonstrate that the first substrate to bind to the enzyme is AdoMet, which is then released as unmethylated form (AdoHcy).

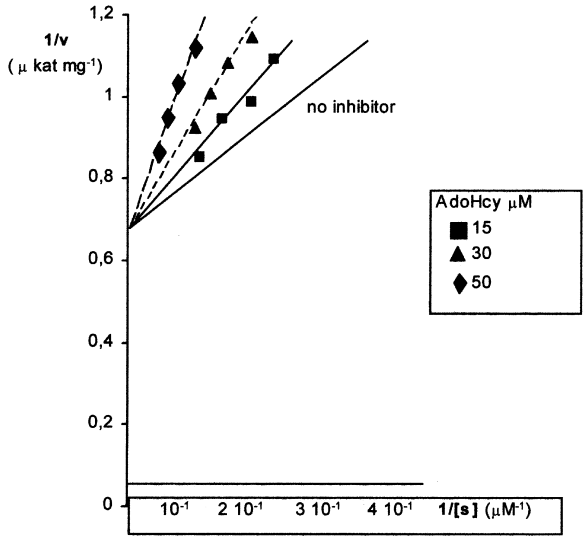


Fig. 9. Double-reciprocal plot of inhibition kinetic of *S*-adenosyl-L-homocysteine (AdoHcy). Lineweaver–Burk plot of $1/v$ vs. 1/[s] (where s = kaempferol) in the presence of different fixed concentrations of *S*-adenosyl-L-methionine. Enzyme concentration held constant at 2 μM. The points are experimental values, and lines were fitted to points by linear regression.

F 4′-OMT crude activity within plant tissues. The results obtained are summarized in Table 3. In the healthy tissues of both *in vivo* plants and *in vitro* explants the detected enzymatic activity was weak and did not change statistically along the 72 h of the observation period. A statistically significant increase of the F 4′-OMT activity could be recorded in the same observation period in the *Fod*-inoculated carnation tissues, both *in vivo* and *in vitro*: the enzymatic activity in the inoculated material increased four times from 24 to 72 h of the observation period, and was more remarkable in the *in vivo* than in the *in vitro* tissues. Figure 11 shows a typical HPLC chromatogram with the initial kaempferol substrate (t_R 2.03 min) used to quantify routinely the F 4′-OMT activity on the base of the amount of its kaempferide methylated derivative (t_R 4.68 min) formed in the course of time.

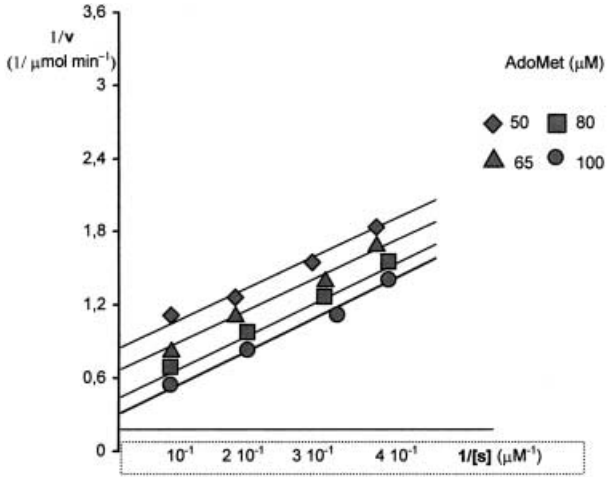


Fig. 10. Lineweaver–Burk plot of F 4′-OMT activity for kaempferol at different concentrations of *S*-adenosyl-L-methionine. Points are experimental values and lines were fitted to points by linear regression.

Table 3. *S*-Adenosyl-L-methionine:flavonoid 4′-*O*-methyltransferase crude activity in healthy and inoculated tissues of the carnation cultivar 'Novada'. In each row, values followed by a same number of * are not statistically different ($P > 0.05$), according to the Student–Neumann–Keuls method. Activity was measured using 100 μM kaempferol as substrate at saturating concentrations of *S*-adenosyl-L-methionine and expressed as nkat·mg protein^{-1}. Values are the mean of 10 different measurements.

	4′-OMT activity measured after hours			
Plant material	0	24	48	72
in vivo Healthy plants[a]	0.02*	0.02*	0.03*	0.05*
in vivo Inoculated plants		0.2*	0.5**	0.8***
in vitro Healthy tissues[a]	0.04*	0.05*	0.05*	0.05*
in vitro Inoculated tissues		0.1*	0.3**	0.4**

[a] Inoculated with sterile water as a control.

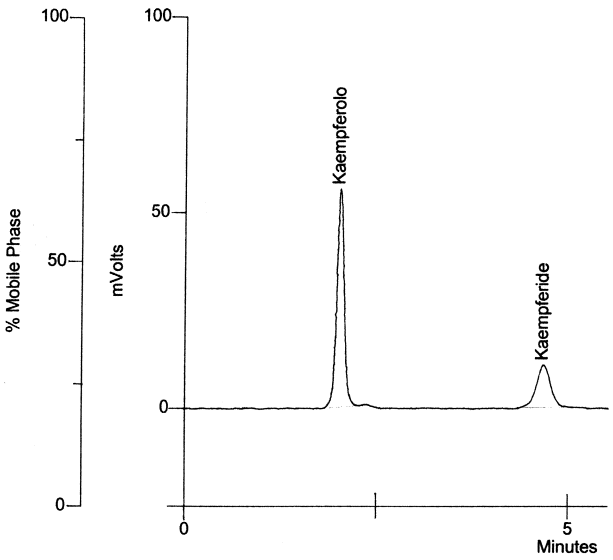


Fig. 11. HPLC chromatogram with the peaks of the initial substrate kaempferol (V) (t_R 2.03 min) and its methylated form kaempferide (XIII) (t_R 4.68 min), obtained through the flavonoid 4′-OMT activity.

F 4′-OMT divalent cation requirement and effect of SH-group reagents. Divalent cations do not appear to be required by the enzyme for its activity (Table 4). An excessive amount (10 mM) of Ca^{++} and Mg^{++} actually depresses the enzymatic activity, while the inhibitory effect of Mn^{++} is already appreciable at 1 mM concentration. The assayed SH-group reagents were strong inhibitors starting from 1 mM concentration.

Table 4. Effect of divalent cations and SH-group reagents on *S*-adenosyl-L-methionine:flavonoid 4′-*O*-methyltransferase activity. Enzyme activity measured in the presence of the different added factors, and expressed as relative to that of controls that did not receive additions.

Additions	Concentration (mM)	Relative activity (%)
None	–	100
Ca^{++}	1	100
Ca^{++}	10	81
Mg^{++}	1	100
Mg^{++}	10	73
Mn^{++}	1	79
Mn^{++}	10	43
Iodoacetamide	1	40
Phenylmercuric acetate	1	20

pH effect. The enzyme activity was evaluated at different pH values using buffer A adjusted at the needed values. The optimum pH value was found around neutrality (pH from 6.9 to 7.0), while the enzyme activity was halved at pH 5.5 and 8.5, and at pH 5.0 it dropped to 4% of the optimal value.

Discussion

An unspecific OMT activity has been reported in carnation tissues [4], but the results of these previous investigations only concerned a 'crude' enzymatic activity. As we have here reported the isolation of a new strictly specific F 4′-OMT from carnation tissues, it is likely that this enzyme could represent only one of the many different OMTs present in the tissues of this ornamental. On the other hand, several distinct OMTs may coexist in plant tissues, originating a multienzyme system which catalyses the methylation sequence of flavonoids [2]. F 4′-OMT shows a high specificity for the flavonoid skeleton, where it methylates exclusively the hydroxy substituent in 4′-position in the presence of the suitable methyl donor, AdoMet; methylation takes place only when the contiguous 3′-position is free. Likewise, other highly specific OMTs have been found in plant tissues, such as the flavonol 8-OMT from *Lotus corniculatus* [19] and the quercetin 3-OMT from apple [20]. When the enzyme specificity is so high, its methylating ability is not confined to the aglycone substrate but may also affect the corresponding glycoside [2]. In the case of the carnation F 4′-OMT, the high affinity towards the flavone kaempferol (V) ($K_m = 1.7$ μM) makes the methylation occur even when sugars are bound to the aglycone, as in kaempferol triglycoside (VI). It is interesting, moreover, to remark that this enzyme is even able to methylate the 4′-position of isoflavones, although at a low rate – an activity unusual for an OMT [21]. This seems to indicate that the presence of a hydroxy substituent in 4′-position is the most important requirement for the enzymatic activity. Actually, when this requirement is satisfied, F 4′-OMT is able to utilize, as well as flavones and flavanones, the isoflavone structure,

just as reported for the *Zea mays* 3′-OMT [22]. In spite of its high selectivity in the catalyzed methylation, this enzyme possesses some characteristics that are commonly shared by other previously described OMTs. In fact, F 4′-OMT consists of a single subunit, as reported for many other plant OMTs [23]. Moreover, its low molecular mass is close to the values reported for several other OMTs [20,24,25]; its pI of 4.15 appears to be lower than the value determined for the flavonol 8-OMT from *Lotus corniculatus* [19] but almost the same found for the *Citrus* 4′-OMT [14]. F 4′-OMT, like other small molecular mass plant OMTs [23], does not require Mg^{++} for its catalytic activity. The specific activity of the pure enzyme is in the range reported for small OMTs [23,26], while its inactivation by –SH group reagents indicates the presence, in the molecule, of essential cys residues, suggesting that carnation F 4′-OMT is a thiol enzyme. There are further important features of this enzyme that deserve to be mentioned: (a) its inability to act on hydroxycinnamic acids to give methoxylated monolignols that are involved in lignin biosynthesis [27]; (b) its activation by the presence of *Fod* within plant tissues; (c) its high substrate affinity towards kaempferol (V), that represents the precursor of the antifungal kaempferide triglycoside (VI) detected in the carnation cultivar 'Novada' [7]. These peculiarities show that the enzyme could have a defensive, rather than a structural, role in plant tissues, where it participates in the formation of methylated flavonoids. It is therefore likely that in carnation, an F 4′-OMT could be involved in the production of a specific methylated flavonoid phytoalexin, just as reported to occur in barley [28]. In several plants, an accumulation of methylated flavonoids has been explained as a protection against pathogens, predators and ultraviolet radiation [2]. A recent investigation concerning the pathogenic interaction cotton × *Verticillium dahliae*, however, reports that the specific pathogen-induced OMT activity is not beneficial to plant defense, as it may impair the phytoalexin defensive system [29]. This points out the complexity of functions of OMT activities, that are associated to several different aspects of plant metabolism and therefore need further specific investigations. With respect to the F 4′-OMT described in this study, experiments are in progress to assay its activity towards an open hydroxychalcone structure and to analyse other fundamental aspects of the enzyme–substrate interactions.

Acknowledgements

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