DMD Fast Forward. Published on November 9, 2011 as doi: 10.1124/dmd.111.041871

Methylation of Catechins and Procyanidins by Rat and Human Catechol-*O*-Methyltransferase: metabolite profiling and molecular modeling studies

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Running title: Methylation of Catechins and Procyanidins in vitro

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Document statistics:

Number of text pages:	26
Number of tables:	3
Number of figures:	4
Number of references:	38
Words in the abstract:	208
Words in the introduction:	568
Words in the discussion:	1516

Non-standard abbreviations:

3'-MeCAT, 3'-*O*-methyl-(+)-catechin; 3'-MeEC, 3'-*O*-methyl-(-)-epicatechin; 4'-MeCAT, 4'-*O*-methyl-(+)-catechin; 4'-MeEC, 4'-*O*-methyl-(-)-epicatechin; APCI, atmospheric pressure chemical ionization; CAT, (+)-catechin; COMT, catechol-*O*-methyltransferase; DHBA, 3,4-dihydroxybenzoic acid; EC,(-)-epicatechin; ESI, electrospray ionization; SAM, S-adenosyl-L-methionine.

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Abstract

Catechins and procyanidins are major polyphenols in plant-derived foods. Despite intensive studies in recent years, neither their biochemical nor their toxicological properties have been clarified sufficiently. This study aimed to compare the methylation of catechins and procyanidins by the enzyme catechol-*O*-methyltransferase *in vitro*. We conducted incubations with rat liver cytosol and human placental cytosol including S-adenosyl-L-methionine (SAM). The set of substrates comprised the catechins (-)-epicatechin (EC) and (+)-catechin (CAT), the procyanidin dimers B1, B2, B3, B4, B5 and B7 as well as procyanidin trimer C1. After extraction, metabolites were analyzed by means of LC-ESI-MS and LC-APCI-MS. EC and CAT were converted to two monomethylated metabolites each by human and rat COMT, with the 3'-*O*-methyl derivatives being consistently the main metabolites. Furthermore, the flavanyl units of procyanidins were methylated consecutively, leading to mono- and dimethylated dimeric metabolites as well as mono-, di- and trimethylated C1 metabolites. The methylation status of each flavanyl unit was determined by means of mass spectrometric quinone-methide fragmentation patterns. In addition, molecular modeling studies were performed with the aim to predict the preferred site of methylation and to verify the experimental data. In conclusion, our results indicate that the degree and position of methylation depends clearly on the three-dimensional structure of the entire substrate molecule.

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Introduction

Flavonoids are a widely occurring class of natural polyphenolic compounds. Their typical C_6-C_3-C_6 backbone includes two aromatic (A, B) and a heterocyclic pyran (C) ring. According to the pyran ring's substitution pattern and oxidation status, six classes of flavonoids are distinguished. Flavonoids with a saturated C-ring bearing one or two hydroxy groups **(Figure 1)** are termed flavanols. The bioavailability and metabolism of monomeric flavan-3-ols, also referred to as catechins, were studied intensively during recent years. However, very often oligo- or polymeric proanthocyanidins constitute the bulk of the flavanol fraction in plants and plant-derived foods. About 15 proanthocyanidin subclasses have been identified among which the procyanidins are the most prevalent representatives. Procyanidins consist of 3',4',3,5,7-pentahydroxylated flavanyl, i.e. catechin units linked in different ways. B-type procyanidins are connected by one carbon-carbon linkage between C-4 and C-8 or C-4 and C-6, whereas doubly linked A-type procyanidins have an additional ether bond between C-2 and C-7. The trimeric C-type procyanidins resemble their dimeric B-type analogs but are elongated by an additional flavanyl unit (Ferreira and Slade, 2002; Beecher, 2003; Gu et al., 2004; Aron and Kennedy, 2008).

Many plant foods are natural sources rich in flavanols, the most important being green tea, wine, cocoa, chocolate, apples, and grapes. Recent analyses even suggest that procyanidins are the main flavonoids in Western diets (Hammerstone et al., 2000; Gu et al., 2004; Manach et al., 2004). Furthermore, catechins and procyanidins are supposed to act as natural antioxidants and vasoprotective agents and to be associated with several other beneficial health effects (Santos-Buelga and Scalbert, 2000; Aron and Kennedy, 2008). Due to their biological potency and high abundance in plant foods catechins and procyanidins moved into the center of research interest during the last two decades.

Several studies investigated the metabolic fate of catechins and procyanidins *in vivo*. It was shown that catechins can cross the gastrointestinal barrier of rats and humans and undergo extensive methylation, glucuronidation and sulfation (Okushio et al., 1999; Baba et al., 2001; Natsume et al., 2003; Tsang et al., 2005). In contrast, recent studies indicate that procyanidins have a very low bioavailability and

neither glucuronides nor sulfates have been detected so far (Holt et al., 2002; Sano et al., 2003). However, small amounts of methylated procyanidin metabolites were observed in rats (Spencer et al., 2001b; Baba et al., 2002; Shoji et al., 2006).

Catechol-*O*-methyltransferase (COMT) is being expressed in numerous mammalian tissues and catalyzes the *O*-methylation of endogenous as well as xenobiotic compounds including flavonoids. In general, the enzyme methylates molecules possessing a catecholic moiety preferentially in the *meta* (3'-*O*-) position (Mannistö and Kaakkola, 1999). Zhu et al. characterized the methylation of catechins by COMT *in vitro* and observed two monomethylated metabolites (Zhu et al., 2000; Zhu et al., 2001) without examining their exact structure. To our knowledge, the methylation of procyanidins has not yet been investigated comprehensively *in vitro*.

Recognizing this particular lack of experimental data, we examined the structure of methylated catechin and procyanidin metabolites formed *in vitro*. For this purpose, we conducted incubations with the catechins (-)-epicatechin (EC) and (+)-catechin (CAT) as well as the B-type procyanidin dimers B1, B2, B3, B4, B5, B7 and the trimer C1. The products were analyzed by means of high performance liquid chromatography and mass spectrometry to elucidate the site of methylation. Additionally, we assessed possible interspecies differences by use of rat and human cytosol. The results were verified by molecular modeling calculations.

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Methods

Chemicals. (-)-Epicatechin and (+)-catechin were purchased from Sigma-Aldrich (Steinheim, Germany). The procyanidins B1, B2, B3, B4, B5, B7 and C1 were isolated by countercurrent chromatography as recently published (Esatbeyoglu and Winterhalter, 2010; Esatbeyoglu et al., 2010). 3'-*O*-methyl-(+)-catechin (3'-MeCAT), 3'-*O*-methyl-(-)-epicatechin (3'-MeEC), 4'-*O*-methyl-(+)-catechin (4'-MeCAT) and 4'-*O*-methyl-(-)-epicatechin (4'-MeEC) were chemically synthesized and characterized as described previously (Donovan et al., 1999b). S-Adenosyl-L-methionine chloride was purchased from Sigma-Aldrich (Steinheim, Germany) or Chromadex Inc. (Irvine, USA). LC/MS-grade solvents (acetonitrile, methanol) were obtained from Fisher Scientific (Loughborough, UK). Ethyl acetate and formic acid were purchased from Acros Organics (Geel, Belgium). All other chemicals were of analytical grade or higher.

Preparation of human and rat cytosolic fractions. Livers from female Unilever-Wistar rats were kindly supplied by the Max Rubner Laboratory of the German Institute of Human Nutrition (Nuthetal, Germany). On the day of preparation, the livers were thawed and weighed. All further steps took place on ice. Portions of 4-5 grams were thoroughly minced with scissors and afterwards suspended in a threefold volume (w/v) of ice-cold buffer (50 mM Tris-HCl, 150 mM KCl, 1 mM EDTA, pH 7.4). Homogenization was carried out with an Ultra Turrax T25 basic homogenizer at 11.000 rpm (6-10 bursts). Tissue homogenates were centrifuged (9.000 *g*, 20 min, 4°C). The supernatants were combined (S9-Mix) and centrifuged for 1 h at 100.000 *g* and 4°C. The latter supernatant, the cytosolic fraction, was split up in aliquots, frozen in liquid nitrogen and stored at –80°C.

Approx. 4 gram of placental tissue from a caucasian woman undergoing caesarean section was kindly supplied by Dr. S. Michaelis of the Benjamin Franklin University Medical Center (Berlin, Germany). The tissue sample was thawed and washed with a surplus of ice-cold PBS to remove blood. Afterwards, the piece of tissue was drained well, weighed, ground with mortar and pestle and suspended in a threefold volume (w/v) of ice-cold buffer. Finally, the minced tissue was homogenized with a potter homogenizer (Schütt homgen plus) at 1500 rpm for 4 min. The centrifugation steps were

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carried out as described above. The protein content of both cytosolic fractions was determined by the method of Lowry (Lowry et al., 1951) with bovine serum albumin as the reference protein. The procedure for procurement of the human placental tissue sample used in this study was approved by the ethics commission of the University of Potsdam.

COMT activity in human and rat placental cytosol. Reaction mixtures contained 200 µM DHBA, 5 mM magnesium chloride, 1 mM dithiothreitol and 300 µM SAM in a final volume of 120 µl. Because of large differences in COMT activity, the protein content was chosen to be 0,1 mg/ml and 8 mg/ml using rat and human cytosol, respectively. After 30 minutes, aliquots of 100 µl were removed and immediately mixed with 100 µl of ice-cold methanol. After centrifugation (10 min, 16100 *g*, 4°C), 180 µl of the supernatant were diluted with 180 µl of 0,1 M ammonium acetate buffer (pH 5). 20 µl of the solution were injected into a Shimadzu HPLC LC-10A series system equipped with a ProntoSIL C18 ace-EPS column (150 mm x 3 mm, 3 µm; Bischoff Chromatography, Leonberg, Germany). The eluents were deionized water containing 0.1% formic acid and acetonitrile. DHBA and its metabolites were monitored at 260 nm and quantified via external calibration curves. Experiments were performed in triplicate.

COMT-catalyzed O-methylation of catechins and procyanidins. The reaction mixtures contained 10 mM potassium phosphate buffer (pH 7.4), 5 mM magnesium chloride, 1 mM dithiothreitol and 300 µM SAM in a final volume of 350 µl. The protein concentration was chosen in according to the estimated reaction rate. We used 1 mg/ml of rat liver cytosolic protein to methylate catechins and procyanidin dimers and 3 mg/ml to methylate trimer C1. In case of controls, cytosolic protein was inactivated by heat (5 min at 95°C) or no SAM was added. After two min of pre-incubation, the reaction was initiated by addition of the substrate (final concentration 50 µM), leading to a methanol concentration of 1% in all reaction mixtures. Sample tubes were gently shaken at 37°C during reaction, and direct light exposure was avoided. After 0, 10, 20, 40, 60 and 90 min, aliquots of 50 µl were removed and immediately extracted twice with 300 µl ethyl acetate. The pooled organic phases

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were evaporated to dryness and residues dissolved in 100 µl of the aqueous eluent. 60-90 µl were injected into the LC/MS system. Reaction mixtures with human placental cytosol contained 8 mg/ml cytosolic protein in a final volume of 120 µl. Aliquots of 50 µl were removed after 0 and 120 min and extracted three times with 300 µl ethyl acetate and further prepared as previously described. All reactions were conducted in triplicate and the extracts were analyzed using the LC/MS system described below.

LC/MS analysis. The LC/MS system (LC-20A prominence, Shimadzu Corporation, Kyoto, Japan) consisted of a SIL-20AC controller with a refrigerated autosampler, two LC-10ADvp pump modules, a DGU-20A5 degasser, a CTO-20AC column oven and a SPD-M20A diode array detector followed by a LCMS-2010 EV single quadrupole mass spectrometer equipped with an ESI- or an APCI-source. The separation was carried out on a PerfectSIL Target ODS-3 column (250 mm x 3 mm, 3µm; MZ Analytical, Mainz, Germany) at a flow rate of 0.3 to 0.4 ml/min. We used water/acetonitrile (95:5, v/v) as the aqueous and acetonitrile or acetonitrile/methanol (50:50, v/v) as the organic eluent. All eluents were acidified with 0.1% formic acid. Gradients were optimized for the best separation of each substrate and its metabolites. Chromatograms were recorded at 280 nm and substances quantified via external calibration curves. Methylated metabolites were quantified as equivalents of the native catechins or procyanidins.

The APCI source was operated in negative-ion mode and the following settings were chosen to yield maximal ion intensity: Scan- or SIM-mode; interface temperature 450 or 500°C; heat block temperature 200°C; curved desolvation line temperature 230°C; nebulizer gas flow 2.5 l/min nitrogen; detector voltage 1.7 kV. The ESI source was used occasionally to detect the quasi-molecular ions unambiguously. It was operated in negative-ion mode (Scan mode; nebulizer gas flow: 1.5 l/min nitrogen).

Molecular modeling experiments. Molecular docking and energy minimization experiments were performed on a Workstation with Dual Xenon Quad Cores using the Molecular Operating

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Environment molecular modeling program (MOE, version 2008.10; Chemical Computing Group: Montreal, Quebec, Canada, 2008).

Protein Structure Preparation. Interactions of the procyanidins B2, B5 and C1 with the COMT enzyme were investigated using X-ray structures deposited in the Protein Data Bank (PDB) under the PDB codes 2ZVJ and 3BWY (Rutherford et al., 2008; Tsuji et al., 2009). Both COMT structures were prepared for docking studies by adding hydrogen atoms and partial charges to the enzyme using the Protonate3D application of MOE2008.10. The charge of the SAM molecule was corrected.

Ligand Structure Optimization. The initial structures of the procyanidin ligands B2, B5 and C1 were built using ChemOffice Suite 2010 and transferred to a MOE database. Molecular mechanical energies of the different conformers of each procyanidin ligand were minimized until a root mean square deviation (RMSD) of 0.01 kcal/mol Å was reached. Energy minimization was performed using the MMFF94 force field option (Halgren, 1996) with the restriction to preserve original chirality of the molecules.

MOE docking. To validate MOE-Dock, we first tested its ability to predict the native binding modes of the known ligands as defined in the crystallographic structures of 2ZVJ and 3BWY (Rutherford et al., 2008; Tsuji et al., 2009). Initial docking runs under standard conditions in MOE lead to insufficient results since re-docking of the original X-ray ligand to its corresponding protein binding site failed. However, under optimized conditions using Pharmacophore Query as well as force field refinement parameters in the MOE Docking panel, MOE-Dock was able to reproduce the crystallographic binding poses. Final docking studies were performed using a model based on the PDB crystal structure 3BWY for human COMT protein. The Sitefinder module of MOE was used to define the catalytic site of the protein for the docking experiments. With respects to the ligands in the binding pocket rotations of the whole molecule as well as rotations around single bonds were allowed with the restriction to retain original configuration. Following these docking studies, we calculated free binding energies of the *meta* hydroxy groups in the B-rings within the COMT active site allowing for different conformations and orientations of the procyanidin molecules. The resulting values were expressed as ranked docking scores (S, in kcal/mol, Table 3).

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In the Pharmacophore Query (see supplemental figure 1S) the radius of Mg^{2+} and the methyl group of SAM were defined as 2.4 Å (property: essential; expression: ML) and 2.8 Å (expression: any), respectively. The aromatic ring was used as Feature 3 with a radius of 2.0 Å (expression: aro). Further details and settings used in the modeling process are given in the supplemental data.

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Results

Methylation of the model substrate DHBA. Using rat or human cytosol, the methylation of DHBA resulted in the formation of two metabolites. The main metabolite was 4-hydroxy-3-methoxybenzoic acid (vanillic acid) in both cases as confirmed by retention time comparison with the analytical standard. The *meta/para* ratio of the DHBA metabolites was approx. 4.5 in both cases. The methylation of DHBA was used to estimate COMT activity in both cytosols. Conditions were chosen to maintain a constant formation rate of vanillic acid for at least 30 min. The COMT activities of rat liver and human placental cytosol were found to be 3972 ± 123 pmol × mg^{-1} × min^{-1} and 65 ± 1 pmol × mg^{-1} × min^{-1}, respectively.

Methylation of catechins using rat liver cytosol. Rat liver cytosolic COMT methylated EC and CAT efficiently. The substrates were converted within 40 minutes, accompanied by the formation of two new peaks in each case. The difference in the mass/charge ratios between the metabolites (m/z 303) and the native catechins (m/z 289) indicated the monomethylation of the catechin backbone. Fragmentation of the quasi-molecular ions was not observed under the conditions used. Using the synthesized reference compounds, the metabolites were identified to be 3'-*O*-methyl and 4'-*O*-methyl derivatives. The earlier eluting 3'-O-methylated metabolites (3'-MeEC or 3'-MeCAT, respectively) were formed predominantly. However, the *meta/para* selectivity was moderate in case of 3'-MeEC and 4'-MeEC (6:1) but more pronounced with respect to 3'-MeCAT and 4'-MeCAT (25:1). Especially 4'-MeCAT was detected only in traces.

Methylation of dimeric procyanidins using rat liver cytosol. Rat COMT methylated all dimeric procyanidins. The change of concentrations over time revealed a biphasic mode of reaction. The conversion of the substrate (m/z 577) coincided with the formation of monomethylated metabolites (m/z 591). After reaching a maximum after 10 to 40 min, the concentration of the monomethylated metabolites declined, accompanied by a continuous increase in the concentrations of dimethylated procyanidin metabolites (m/z 605).

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Using the APCI ion source, fragmentation of procyanidin dimers and their methylated analogs was observed **(Figure 2)**. The cleavage of the native procyanidin dimers ($[M-H]^-$, m/z 577) led to the formation of two fragments with m/z 287 and m/z 289. Assuming a heterolytic cleavage of the interflavan linkage (Karchesy et al., 1989), these fragments represented the first and the second flavanyl unit, respectively. In case of the monomethylated procyanidin dimers ($[M-H]^-$, m/z 591), two different fragmentation patterns were observed. The presence of fragments with m/z 301 and 289 indicated the methylation of the first flavanyl unit, whereas the occurrence of fragments with m/z 287 and 303 suggested the presence of a methylated second flavanyl unit. Furthermore, if two monomethylated metabolites showed the same pair of fragment ions (301/289 or 287/303), they were considered to be a pair of *meta* and *para* isomers because the same flavanyl unit had obviously been modified in both cases. Such pairs of *meta* and *para* isomers were observed among the monomethylated metabolites of all investigated dimeric procyanidins. Unfortunately, the quinone-methide fragmentation pattern did not allow a distinction between the *meta* and the *para* isomer. Therefore, the exact *meta/para*-ratio of the respective isomers could not be determined but differences in relative abundance were taken as an indication for increased or decreased *meta/para*-selectivity in comparison with the free catechins (see supplemental data). In contrast, all dimethylated metabolites revealed the same quasi-molecular (m/z 605) and fragment ions (m/z 303, m/z 301), showing the monomethylation of each subunit. The absence of fragments with m/z 316 and 318 in the spectra of all procyanidin metabolites showed that dimethylation of one flavanyl unit never occurred.

Table 1 summarizes the metabolite profiles of all investigated catechins and dimeric procyanidins. Among the pairs of monomethylated procyanidin metabolites with a methylated first flavanyl unit, one emerged clearly as main metabolite but the ratio between these two metabolites differed substantially. In contrast, monomethylated procyanidin metabolites bearing the methylation in the second flavanyl unit were found, if detected at all, in much lower concentrations (see supplemental data). In some cases, the existence of further minor metabolites was assumed. Representative chromatograms of the extracted aliquots are given in **Figure 3.**

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Methylation of the trimeric procyanidin C1 with rat liver cytosol. The methylation of procyanidin trimer C1 by rat liver cytosolic COMT yielded five monomethylated, nine dimethylated and four trimethylated metabolites as confirmed by ESI-MS. The presence of two interflavan linkages in the molecule enabled two different fragmentation events in the APCI source. The first cleavage of trimer C1 (m/z 865) separated either the first (m/z 287) or the third flavanyl unit (m/z 289) from the remaining dimeric fragment (m/z 577 or m/z 575, respectively). In a second step, the dimeric fragment disintegrated partly into single flavanyl units with the same masses as the fragments resulting from the first cleavage (m/z 287/289 or m/z 287 only, respectively). The C1 metabolites fragmented analogously and the methylation status of each flavanyl unit was determined mainly based on the mass of the quasi-molecular ion and the dimeric fragments resulting from the first cleavage (**Table 2**). Although the second fragmentation step which could not be completely avoided by optimizing APCI settings generated additional fragments, the methylation status of the flavanyl unit could be unequivocally determined in most cases. The site of methylation remained unidentified in case of five minor metabolites because intensity of the decisive fragment ions was too low. Thus, 13 metabolites formed were structurally elucidated and five minor metabolites were identified only as quasi-molecular ions. The number and the structure of the C1 metabolites identified are summarized in **Table 2**. Representative chromatograms of the extracted aliquots are given in **Figure 3.**

Rat liver cytosolic COMT methylated the catecholic hydroxy groups of trimer C1 selectively. The two most abundant metabolites, MeC1 B and Me2C1 G, bore a methyl group on the first or the first and the second flavanyl unit, respectively. Another important metabolite was methylated solely on the second flavanyl unit. Most of the other mono-, di- or trimethylated metabolites reached substantially lower concentrations. In analogy to the procyanidin dimers, it was possible to identify those dimethylated metabolites which form a pair of *meta* and *para* isomers but it was impossible to distinguish between the *meta* and the *para* isomer. However, obviously the *meta/para* ratio of these paired isomers differed considerably (see supplemental data).

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Methylation of DHBA, catechins and procyanidins with human placental cytosol. Because of the low COMT activity of the human placental cytosol, the incubations were conducted with 8 mg protein per ml to yield sufficient conversion rates. Remarkably, the metabolite spectrum generated by human COMT was qualitatively identical with that of the rat enzyme. Furthermore, the different metabolites were formed in approximately the same relative proportions.

Molecular Modeling. Based on the experiments *in vitro*, we chose B2, B5, and C1 as representative procyanidins for molecular modeling studies. We assumed that the respective catecholic functions of each flavanyl unit of the B2, B5 and C1 molecules (see supplemental figures 2S, 4S, and 6S) are able to bind to the catalytic site of the COMT enzyme. Docking calculations confirmed this assumption in general; only in case of the second flavanyl unit of C1 accessibility was reduced due to steric hindrance of the catecholic moiety. Furthermore, the modeling revealed that the catecholic B-rings are the only regions in the particular molecules that fit into the catalytic site of COMT. Additionally, the three-dimensional arrangement of the critical components in the active site strongly preferred the *meta* hydroxy group of a catecholic moiety to form the transition state leading to *O*-methylation of the substrate **(Figure 4)**.

Finally, we aimed to assess the probability of the different flavanyl units of B2, B5 and C1 to undergo a methylation reaction. For that purpose, we evaluated the quality of fit between the *meta* hydroxy groups in the B-rings and the active site of COMT for different poses (conformations and orientations) of the molecules by calculating free binding energies (docking scores, **Table 3**). The 3' hydroxy group of the first flavanyl unit of B2 yielded much better scores than the 3' hydroxy group of the second flavanyl unit. In contrast, the score difference between the first and the third flavanyl unit of C1 was much smaller, and the values for the first and the second flavanyl unit of B5 were comparable. These results provide an explanation for the highly selective methylation of B2 and the comparably unselective methylation in case of B5 (see supplemental data). However, in contrast to the experimental results, no score could be obtained for the second flavanyl unit of C1 due to lack of fit. This is in disagreement with the experimental data which show that a methylation of the second

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flavanyl unit of C1 is possible. The schematic representation of the interaction between the COMT enzyme and the various procyanidins B2, B5, and C1 is given in the Supplemental figures 3S, 5S, and 7S, respectively.

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Discussion

The human and rat COMT enzymes methylated the investigated catechins and procyanidins. Interestingly, the resulting metabolite profiles were identical with respect to the number, structure and relative proportions of the metabolites. The model substrate DHBA and the catechins were converted to two monomethylated derivatives. The catecholic function of DHBA, EC and CAT was preferentially methylated in the *meta* (3') position by human and rat COMT. The observed *meta/para* ratios were approx. 4.5:1, 6:1 and 25:1, respectively.

Our results agree with the main lines of recent reports. Zhu et al. reported the formation of two monomethylated EC metabolites *in vitro* with a ratio of ~5:1 after incubations with human and rat cytosols (Zhu et al., 2000; Zhu et al., 2001). In rat studies and perfusion experiments with rat intestinal models, either 3'-MeEC alone (Baba et al., 2001; Donovan et al., 2002; Natsume et al., 2003) or 3'-MeEC and 4'-MeEC (Okushio et al., 1999; Kuhnle et al., 2000) were detected in biological fluids and intestinal perfusates. Occasionally, glucuronidated forms of 3'-MeEC were also detected in rat plasma, urine and bile (Harada et al., 1999; Okushio et al., 1999; Natsume et al., 2003). We conclude that there is convincing evidence for the hypothesis that human and rat COMT enzymes generate primarily 3'-MeEC. Therefore, one would expect 3'-MeEC rather than 4'-MeEC in humans. However, Natsume et al. identified two different 4'-MeEC glucuronides but no 3'-MeEC glucuronides in human plasma and urine whereas only 3'-*O*-methylation was observed in rats (Natsume et al., 2003). Further investigations are necessary to remove these discrepancies.

We observed that CAT was almost exclusively converted to the metabolite 3'-MeCAT. In several rat and human studies only 3'-*O*-methylated CAT or the respective conjugates were observed *in vivo* or after *in situ* perfusion (Donovan et al., 1999a; Harada et al., 1999; Baba et al., 2001; Donovan et al., 2001; Donovan et al., 2002). Combining these results, it seems very likely that only 3'-*O*-methyl CAT is of importance *in vitro* and *in vivo* because its formation is enzymatically highly favored.

According to our results *in vitro*, human placental and rat liver cytosolic COMT methylated dimeric procyanidins with roughly the same efficiency as catechins. Both flavanyl units were monomethylated consecutively with the first flavanyl unit being predominantly modified in the first step of reaction.

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However, the number and structure of the metabolites as well as the relative amounts in which the individual metabolites were formed differed substantially (**Table 1** and supplemental data). Although not being able to identify *meta* and *para* isomers exactly by means of mass spectrometric fragmentation patterns, we observed that several monomethylated dimeric metabolites obviously belong together as a pair of *meta* and *para* isomers. We made this assignment based on a) the observation that often two monomethylated metabolites were formed which showed identical fragmentation patterns, and b) the generally accepted assumption that COMT methylates only catecholic but never single phenolic hydroxy groups. Considering the ratios in which the respective isomers were formed, the degree of *meta/para* selectivity among these paired isomers appeared to vary widely. For example, selectivity of the initial methylation of the first flavanyl unit was high in case of B1 and B2 and low in case of B3 and B5. Our data also show that there is a difference in *meta/para*-selectivity between the methylation of free catechins and the corresponding flavanyl units of the procyanidins. Additionally, the presence of a (4→6)-bond seems to favor the formation of four dimethylated metabolites, but does not necessarily implicate a higher overall number of metabolites. In conclusion, the spectrum of methylated metabolites cannot be deduced solely on the basis of the sequence of catechin subunits and the type of the interflavan linkage.

The same principles are seemingly valid for the methylation of procyanidin trimer C1. As in case of the dimers, the methylation of the first flavanyl unit occurred early and highly selective. The other subunits were amenable to methylation as well. Altogether, the methylation of C1 is more slowly than the methylation of the dimers. Procyanidins like other compounds with tannin properties can bind to proteins, but these interactions are much more strongly in case of the higher oligomers (Sarni-Manchado et al., 1999). Therefore, we suppose that C1 may affect COMT activity by unselective interaction.

An important aim of our study was to generate the entire set of methylated metabolites of the selected catechins and procyanidins. Thus, to be able to detect and characterize even minor metabolites, we chose a comparatively high substrate concentration of 50 µM. In doing so, we observed for instance the formation of the methylated EC metabolites in almost the same proportions as reported by Zhu et

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al. who used substrate concentrations ranging from 5 to 30 µM (Zhu et al., 2000; Zhu et al., 2001). Although we cannot exclude an influence of the substrate concentration on the *meta/para*-ratio, we suppose it to be of limited extent. Therefore, our results can be helpful for the interpretation of results from studies *in vivo*.

To verify the experimental data, we performed molecular modeling studies with the human COMT enzyme and selected procyanidins. According to the docking studies each B-ring of B2, B5 and C1 can bind to the active site of COMT. Apparently such binding requires the presence of a catecholic group because only this substructure can coordinate the Mg^{2+} ion and form the transition state with Lys144 and SAM **(Figure 4)**. Containing a *meta* and a *para* hydroxy group, a catecholic B-ring is theoretically able to bind to the active site in two different ways. Thus, the number of theoretical binding modes is two per flavanyl unit, i.e. four in case of the procyanidin dimers and six in case of C1. In contrast, the docking studies indicate that the orientation leading to 3'-*O*-methylation is energetically favorable. This conforms to the described binding mode of catecholic substrates to COMT (Palma et al., 2006; Bai et al., 2007) and our results *in vitro* which show that 3'-*O*-methylation of catechins is clearly the major pathway. Therefore, we postulate that procyanidins may also be predominantly 3'-*O*-methylated by COMT.

Moreover, the three-dimensional structure of the entire substrate molecule influences the number of metabolites (see supplemental data). For example, the best docking score for the complex formed between B2 and the active site of the COMT protein (-54.88 kcal/mol) was obtained for the 3' hydroxy group of the B-ring of the first flavanyl unit. This score was much better than the best docking score for the analogous group on the second flavanyl unit (-38.93 kcal/mol; **Table 3**) and explains why, at first, the first flavanyl unit of B2 is methylated exclusively. However, the methylation of the second flavanyl unit of the monomethylated B2 metabolite can occur as the second step. In contrast, the best docking score values for the second (-47.20 kcal/mol) and the first flavanyl unit (-44.66 kcal/mol) of B5 differed much less. This is in line with our experimental data showing that the methylation of B5 occurred less selective. However, the two different 3' hydroxy groups were not methylated to the same extend as the small differences in the docking scores would suggest. In case of

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procyanidin trimer C1, the docking scores support the preferential methylation of the first and the third flavanyl unit. No score could be obtained for the second flavanyl unit due to lack of fit whereas the experimental data provide evidence for the methylation of this flavanyl unit. Apparently, the molecular modeling of the interaction between procyanidin trimer C1 and the COMT enzyme remained partly inconclusive.

Recently, the detection of small amounts of procyanidins in human plasma (Holt et al., 2002; Sano et al., 2003) or procyanidins and their methylated derivatives in rat plasma or intestinal perfusates was reported (Spencer et al., 2001b; Shoji et al., 2006). Despite their low bioavailability, procyanidins are being associated with a variety of beneficial health effects (Aron and Kennedy, 2008), especially a high antioxidative activity. After being transformed into a relatively stable o-semiquinone by radical species, the catecholic B-ring can act as an effective radical scavenger (Bors and Saran, 1987). This effect is especially pronounced in case of the procyanidins because of a phenomenon called phenolic coupling (Bors and Michel, 2002). The biological implications of the methylation of the catecholic B-ring are yet poorly characterized. As expected, the antioxidative capacity of 3'-*O*-methylated EC is reduced with respect to unmethylated EC, but both substances protect cultivated human fibroblasts equally against apoptosis (Spencer et al., 2001a). The very efficient methylation of catechins and procyanidins by COMT might be intended to deactivate the catecholic structure as a potentially reactive center.

In conclusion, we report that catechins and procyanidins were methylated by human and rat cytosolic COMT. Because of the occurrence of one catecholic structure per flavanyl unit, monomers, dimers and trimer C1 underwent mono-, bi- or triphasic reactions, respectively. Among the procyanidins, the number and relative ratio of the metabolites depended on the three-dimensional structure of the entire molecule and not only on the arrangement of the catechin subunits or the type of the interflavan bond. No qualitative differences have been observed between the metabolite profiles of rat and human COMT.

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Authorship Contributions

Participated in research design:	Weinert, Wiese, Kulling
Conducted experiments in vitro:	Weinert
Performed molecular modeling studies:	Homann
Contributed analytical reference compounds:	Esatbeyoglu, Winterhalter
Performed data analysis:	Weinert
Contributed to the writing of the manuscript:	Weinert, Wiese, Homann, Rawel, and Kulling

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DMD Fast Forward. Published on November 9, 2011 as DOI: 10.1124/dmd.111.041871
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Footnotes

The work was supported by the German Federal Ministry of Education and Research (BMBF) within the scope of the joint project “Dietary procyanidins” [Grant 0313828A].

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Figure legends

Fig. 1. Chemical structures of the investigated flavanols. - Catechins (A), (4→8)-linked procyanidin dimers (B), (4β→6)-linked procyanidin dimers (C) and the (4β→8)-(4β→8)-linked procyanidin trimer C1 (D).

Fig. 2. Fragmentation of dimeric native and mono- or dimethylated procyanidin quasi-molecular ions in the APCI ionsource. – For clarity, only the probably more dominant *meta*-methylation of the catecholic groups is shown. FU, flavanyl unit.

Fig. 3. Representative HPLC chromatograms (recorded at 280 nm) of the extracted aliquots of the B2 (A), B5 (B) and C1 incubations (C) with rat liver cytosol. – Monomethylated, dimethylated and trimethylated metabolites are designated M, D and T, respectively, and numbered for each procyanidin substrate according to the order of elution. MP, matrix peak.

Fig. 4. Molecular modeling of the interaction between B2 (magenta) with the amino acid residues of COMT in the substrate binding pocket. The COMT protein is depicted in ribbon representation and colored by different secondary structures. The water molecule and the Mg^{2+} ion are colored cyan. The SAM is in yellow. Hydrogen bonds are indicated by an orange dashed line. The distance between main factors in catalytic core are given in Å and colored green. Interaction between the reacting hydroxy group for methylation of B2 and Lys 144 enables the H^+ transfer to activate the catechol.

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Tables

Table 1. Number and structures of the methylated catechins and dimeric procyanidin metabolites.

– FU, flavanyl unit.

Compound	Structure			Monomethylated metabolites		Dimethylated metabolites	Overall number of detected metabolites
	FU1	Linkage	FU2	FU1	FU2		
EC	EC	---	---	2	---	---	2
CAT	CAT	---	---	2	---	---	2
B1	EC	(4β→8)	CAT	2	1	3	6
B2	EC	(4β→8)	EC	2	---	3	5
B3	CAT	(4α→8)	CAT	2	1	3	6
B4	CAT	(4α→8)	EC	2	1	3	6
B5	EC	(4β→6)	EC	2	2	4	8
B7	EC	(4β→6)	CAT	2	---	4	6

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Table 2. Fragmentation of the observed mono-, di- and trimethylated metabolites of procyanidin C1 in the APCI source. – The structure of five minor metabolites remained unclear. FU, flavanyl unit.

Compound name	Position of methyl group	Molecular ion (m/z)	Fragments after 1st cleavage (m/z)				Possible additional fragments after the 2nd cleavage (m/z)	**Number of identified isomers**
			FU1	FU2+3	FU1+2	FU3		
Native C1		865	287	577	575	289	---	**---**
Monomethylated C1	FU1	879	301	577	589	289	287	**1**
	FU2		287	591	589	289	301	**1**
	FU3		287	591	575	303	---	**1**
Dimethylated C1	FU1+2	893	301	591	603	289	---	**2**
	FU2+3		287	605	589	303	301	**2**
	FU1+3		301	591	589	303	287	**2**
Trimethylated C1	FU1+2+3	907	301	605	603	303	---	**4**

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Table 3. Calculated docking scores (expressed as binding free energy S in kcal/mol) for different poses for the three tested ligands B2, B5, and C1 to human COMT. * Values in bold represent the fit with the best docking score for the respective flavanyl unit (FU1, FU2, FU3). The calculation of the ligand-COMT binding free energies was conducted by including solvation effects computed with the generalized-Born model. Please refer to the Supplementary figures 2S, 3S, and 5S for illustration of the flavanyl units FU1, FU2 and FU3 in each molecule.

Procyanidin ligand	S (kcal/mol)	Methylation in *meta* position of the named flavanyl unit (FU)
B2	**-54.88**	**FU 1 ***
	-47.19	FU 1
	-45.49	FU 1
	-38.93	**FU 2 ***
B5	**-47.20**	**FU 2 ***
	-45.67	FU 2
	-45.28	FU 2
	-45.03	FU 2
	-44.66	**FU 1 ***
C1	**-53.94**	**FU 1 ***
	-52.10	FU 1
	-48.53	**FU 3 ***

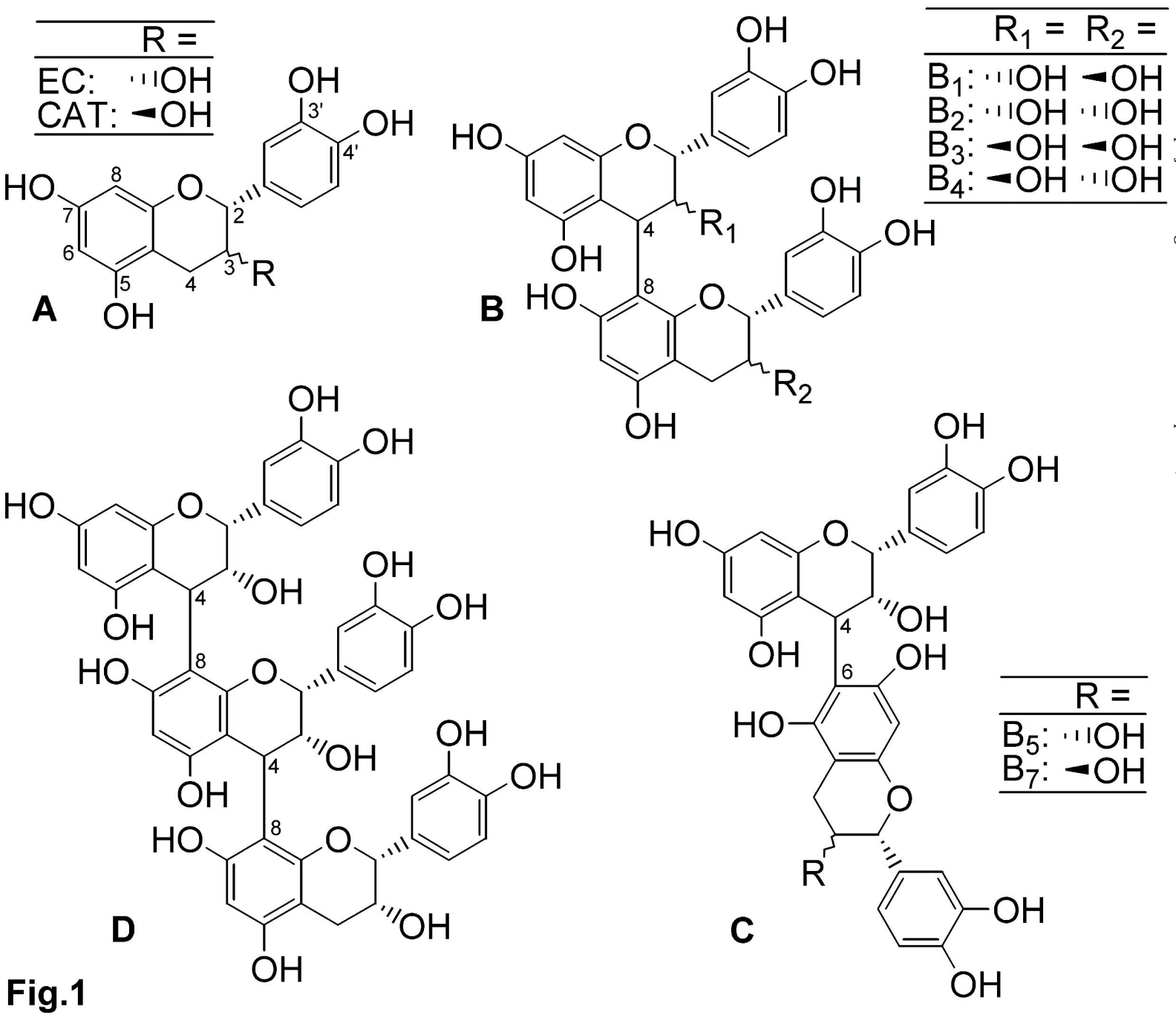
R =
EC: OH
CAT: OH
A
R1 = R2 =
B1: OH OH
B2: OH OH
B3: OH OH
B4: OH OH
B
R =
B5: OH
B7: OH
C
D

Fig.1

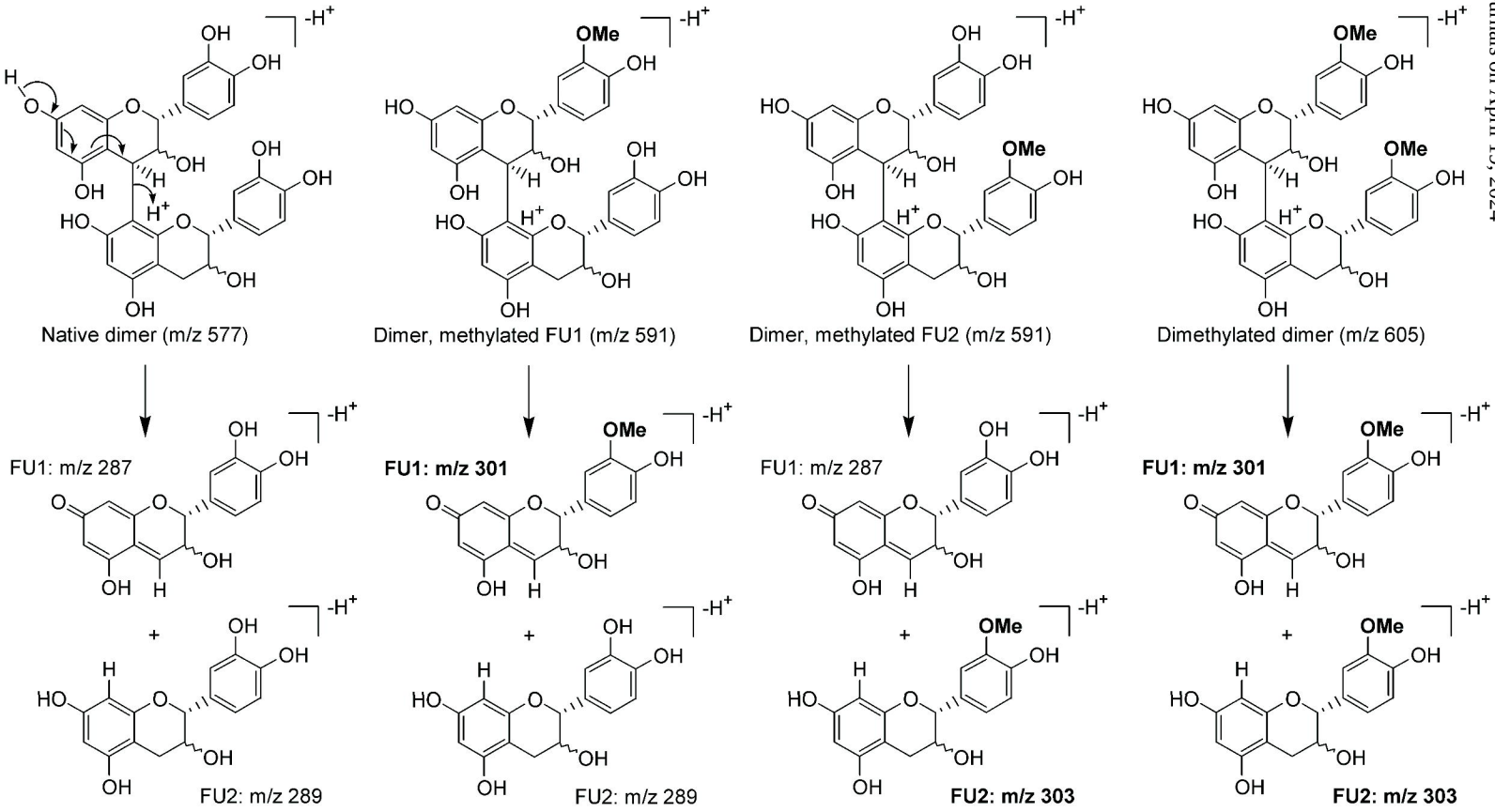
Native dimer (m/z 577)
Dimer, methylated FU1 (m/z 591)
Dimer, methylated FU2 (m/z 591)
Dimethylated dimer (m/z 605)
FU1: m/z 287
FU1: m/z 301
FU1: m/z 287
FU1: m/z 301
FU2: m/z 289
FU2: m/z 289
FU2: m/z 303
FU2: m/z 303

Fig.2

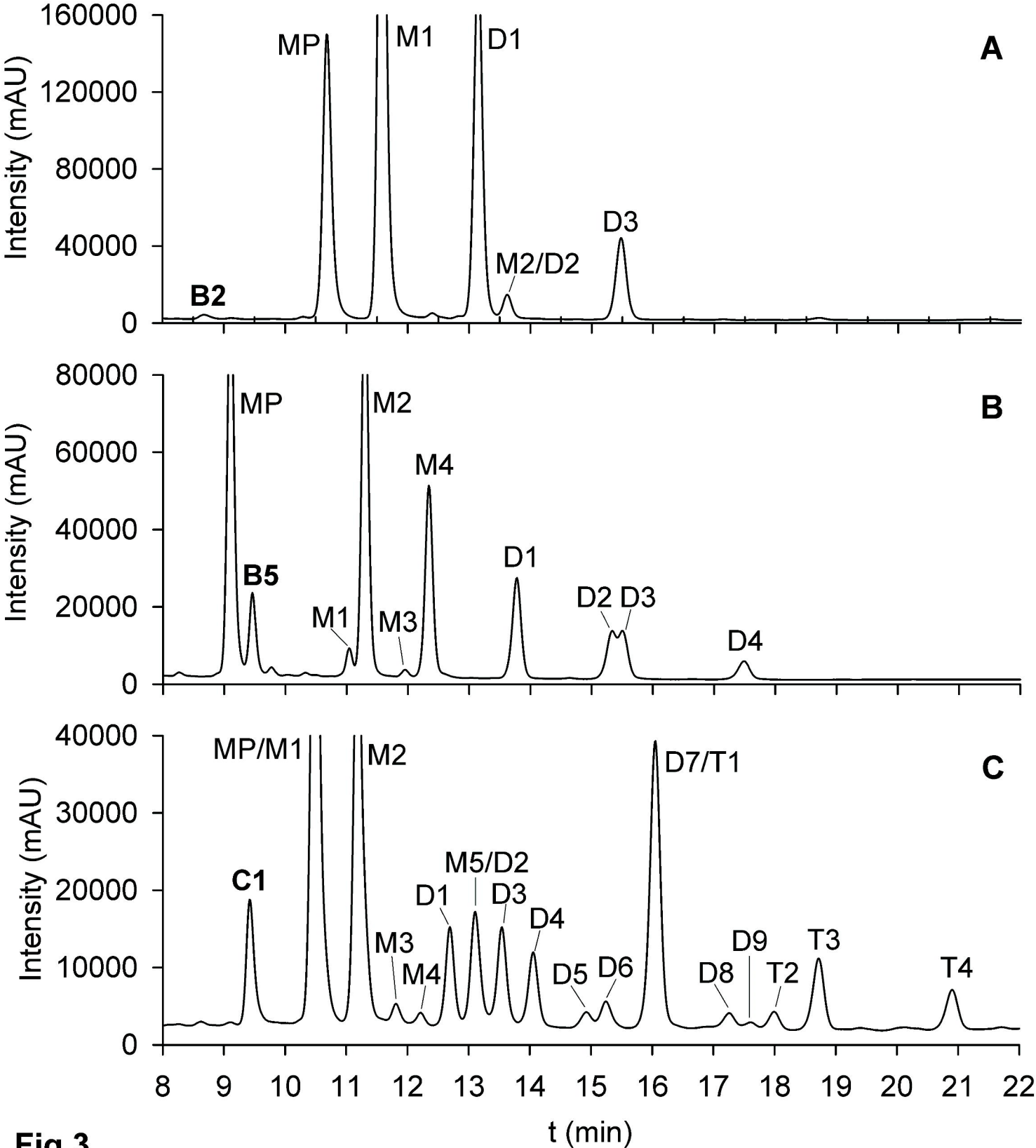
A
MP
M1
D1
D3
M2/D2
B2
Intensity (mAU)
160000
120000
80000
40000
0
B
MP
M2
M4
D1
B5
M1
M3
D2 D3
D4
80000
60000
40000
20000
0
C
MP/M1
M2
D7/T1
C1
M5/D2
D1
D3
D4
M3
M4
D5
D6
D8
D9
T2
T3
T4
40000
30000
20000
10000
0
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
t (min)

Fig.3

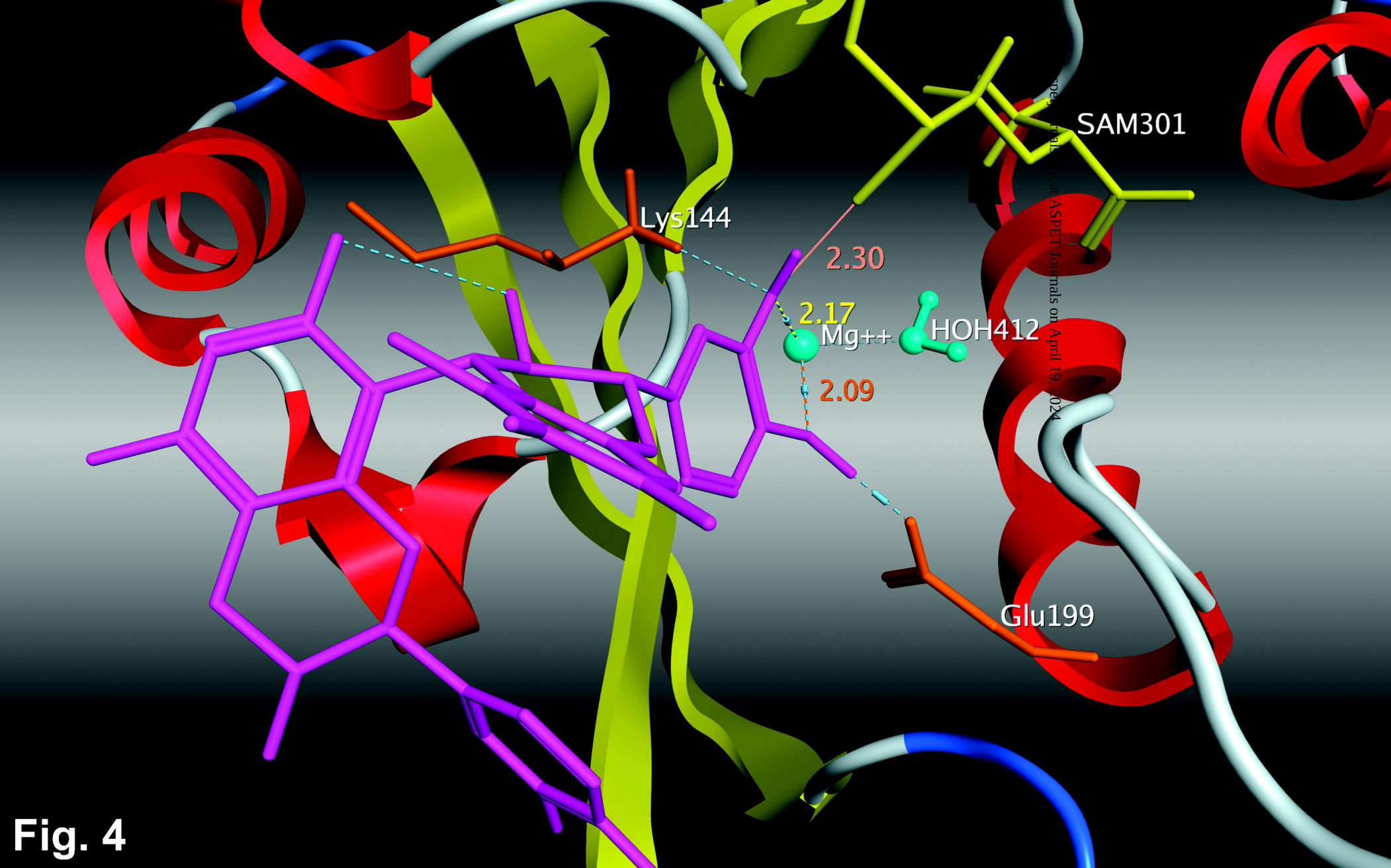

Fig. 4