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Enzymatic Synthesis of [3′-*O*-*Methyl*-^{3}H]malvidin-3-glucoside from Petunidin-3-glucoside

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Malvidin-3-glucoside has been labeled by enzymatic synthesis in a single-step experiment. Catechol-*O*-methyl transferase catalyzed the B-ring methylation of petunidin-3-glucoside, and *S*-Adenosyl-L-[*methyl*-^{3}H] methionine was the methyl donor. Solid phase extraction and preparative high-performance liquid chromatography were necessary to separate [3′-*O*-*methyl*-^{3}H]malvidin-3-glucoside from an isomer and the starting material. The specific activity was 2.2 Ci mmol^{-1}, and the yield of incorporation was 1.1%. A possible application of the labeled material is the study of anthocyanin reactions in complex mixtures such as red wine where products are difficult to isolate and analyze.

KEYWORDS: Malvidin-3-glucoside; catechol-*O*-methyl transferase; *S*-adenosyl-L-[*methyl*-^{3}H]methionine

INTRODUCTION

Anthocyanins are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrilium. These compounds are found in many fruits and flowers, and they provide colorful pigmentation to many plants. Anthocyanins are ingested by humans from fruits, vegetables, or after food processing in such products as red wine or juices. There is also a strong interest in anthocyanins as a source of natural food colorants (*1*).

Malvidin-3-glucoside is one of many anthocyanins found in nature, and it is the major anthocyanin in *Vitis vinifera* grapes and therefore in red wines (*2*). For many years, it has been known that anthocyanin concentration decreases during wine aging (*3*), but the type of reactions, the kinetics, and the reaction products remain to be fully characterized. Radioactivity tracing of anthocyanins can provide a new approach to study the chemical fate of these compounds.

A synthetic method for unlabeled malvidin-3-glucoside was reported in 1931 starting from syringic acid and followed by many steps (*4*). Radiolabeled anthocyanins have been obtained from cell cultures using [1-^{13}C]-L-phenylalanine (*5*) or by incubation of plants with $^{14}CO_2$ (*6*). This type of labeling requires working with living tissues, and the dilution of labeled precursor by endogenous metabolite pools inevitably results in low specific activity.

Catechol-*O*-methyl transferase (COMT) is an enzyme (EC 2.1.1.6) involved in the metabolism of catecholamines as well as detoxification of phenolic compounds coming from plant sources (*7*). The catechol substrate is methylated at a meta or para position relative to a molecular linkage to the main structure. The enzyme requires Mg^{2+} for activity and is commercially available from porcine liver. The methyl donor agent is *S*-adenosyl-L-methionine (AdoMet), which is commercially available with either ^{3}H or ^{14}C in the donating methyl group. Several papers have described the methylation in vitro of different phenolic compounds by this enzyme (*8*−*10*), but there are no reports where anthocyanin has been used as a substrate. Interestingly, Miyazawa and collaborators (*11*) described the methylation in vivo of cyanidin-3-glucoside administered to rats to peonidin-3-glucoside, presumably catalyzed by COMT.

The purpose of this study was to obtain radiolabeled malvidin-3-glucoside with moderately high specific activity, from readily available starting materials, and in the fewest steps possible.

MATERIALS AND METHODS

Chemicals. An amount of 1 mCi of *S*-adenosyl-L-[*methyl*-^{3}H]-methionine contained in a solution of 10 mM H_2SO_4:ethanol (9:1) with a specific activity of 15 Ci mmol^{-1} (555 Gbq mmol^{-1}) was purchased from New England Nuclear (Boston, MA). Tris-buffer, DL-dithiothreitol (DTT), $MgCl_2$, and cathecol-*O*-methyl transferase from porcine liver were purchased from Sigma (St. Louis, MO). Petunidin-3-glucoside and malvidin-3-glucoside were purchased from Polyphenols SA (Sandnes, Norway). Petunidin-3-glucoside contained malvidin-3-glucoside and peonidin-3-glucoside as minor impurities. Ultima Gold (scintillation fluid) and tritiated water, used as internal standard, were purchased from Packard (Meriden, CT).

S-Adenosyl-L-[*methyl*-^{3}H]methionine in sulfuric acid and ethanol solution (0.55 mCi mL^{-1}) was evaporated under N_2 in a water bath at 25 °C to dryness. Immediately after, it was resuspended with 100 μL of 0.327 N NaOH and added to a solution containing 50 μL of 600 mM Tris-HCl buffer (pH 8.3 at 25 °C), 10 μL of 24 mM $MgCl_2$, 10 μL of 80 mM DTT, 30 μL of 12.6 mM petunidin-3-glucoside, and 1000 units of COMT. The reaction mixture was incubated for 1 h at 37 °C, and the reaction was stopped by the addition of 200 μL of 1 N HCl and placement of the reaction vial in ice. The reaction mixture was then added to a C18 solid phase extraction cartridge (100 mg, Supelco, St. Louis, MO) previously conditioned with 2 mL of methanol followed by 2 mL of water. The stationary phase was then washed with 0.12 N HCl(aq), followed by methanol:water (1:4; 0.12 N HCl). Petunidin-3-glucoside and its reaction products were collected with

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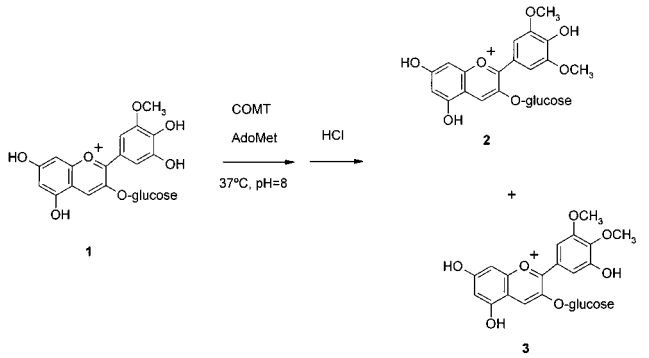

Figure 1. Chemical structure of petunidin-3-glucoside (**1**) and the methylation products obtained after incubation with COMT, malvidin-3-glucoside (**2**) and an isomer (**3**). The flavylium structures shown are only present at low pH.

methanol:water (1:4; 0.12 N HCl). To calculate specific activity and yield of the products, a small fraction (6%) was diluted with water (1:1) and purified by preparative high-performance liquid chromatography (HPLC) with an analytical column. The instrument was a Hewlett-Packard 1090 Series II (Palo Alto, CA). The column was a Luna C18-2 (particle size 5 μm, 250 mm $\times$ 4.6 mm) purchased from Phenomenex (Torrance, CA) and protected with a guard column (4 mm $\times$ 3 mm) containing the same material. Injection volume was 200 μL, and eluent was monitored at 520 and 280 nm. This method uses a binary gradient with mobile phases: (A) trifluoroacetic acid:water (0.25:99.75) and (B) trifluoroacetic acid:water:acetonitrile (0.25:17.75:80). The flow rate was 0.8 mL min^{-1}; linear gradients were from 25 to 40% B for 15 min, 40 to 80% in 0.5 min, 80% for 2 min, followed by reequilibration of the column. Malvidin-3-glucoside (retention time 9.4 min) and the other reaction product (retention time 10 min) were collected with a Gilson fraction collector model 202 (Middleton, WI), collecting fractions every 0.05 min. A 0.1 N HCl solution (350 μL) was added to all of the fractions to increase the sample volume. A small volume from each fraction was then measured for radioactivity and compared to the HPLC chromatogram to determine which fractions contained [3′-*O*-*methyl*-^{3}H]malvidin-3-glucoside.

Analysis. The radioactivity of all samples was determined using a Packard Tri-Carb liquid scintillation analyzer model 2000 (Meriden, CT) by adding 10 mL of scintillation cocktail to all samples. The amount of radioactivity for specific activity calculation was determined by the internal standard method by adding known amounts of tritiated water to the sample to calculate efficiency (*12*).

[3′-*O*-*Methyl*-^{3}H]malvidin-3-glucoside and the isomer concentration were calculated spectrophotometrically based on malvidin-3-glucoside standard in a 0.1 N HCl solution (λ_{max} = 520 nm and ϵ = 36 600 M^{-1} cm^{-1}). The spectrophotometer was a Hewlett-Packard 8452A Diode Array, used with UV−visible ChemStation A.08.03 software.

LC/mass spectrometry was performed on a Hewlett-Packard 1100 Series LC/MSD system with ES/API source in positive mode using the LC conditions described above. Gas temperature was 350 °C, drying gas was 10 L min^{-1}, nebulizer pressure was 60 psi, and capillary voltage was 4000 V.

RESULTS AND DISCUSSION

Initial experiments with unlabeled AdoMet (not shown) indicated that petunidin-3-glucoside can be methylated at the two hydroxyl groups in the catechol group (**Figure 1**). The reaction yields the desired product (malvidin-3-glucoside) and presumably an isomer of malvidin (3-hydroxy 4,5-dimethoxy), a product not described previously in the literature. This isomer has the same mass-to-charge ratio as malvidin-3-glucoside (M^+ = 493) by electrospray mass spectrometry but a different retention time. Assignment of malvidin-3-glucoside was confirmed by coinjection with a pure standard.

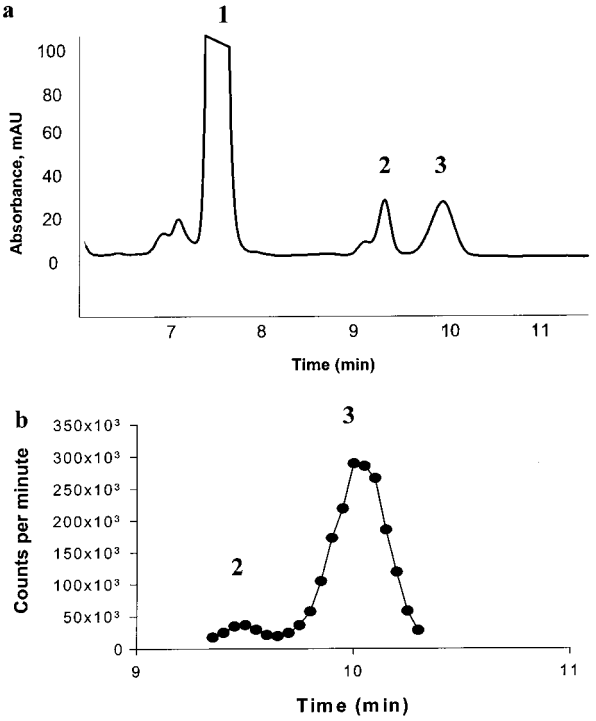

Figure 2. (**a**) HPLC chromatogram at 520 nm of the reaction mixture with petunidin-3-glucoside (**1**) eluting first, followed by malvidin-3-glucoside (**2**) and the presumed malvidin-3-glucoside isomer (**3**). Malvidin-3-glucoside has a shoulder caused by peonidin-3-glucoside. (**b**) HPLC fractions (0.05 min, 0.04 mL) were collected in the region of interest, and the radioactivity was calculated by liquid scintillation counting expressed in counts per minute.

Anthocyanins exist in different forms depending on the pH (*13*). At low pH, the flavylium form is favored, but at pH 8.0 (the reaction pH used here), most of the anthocyanin is in the pseudobase (or carbinol) form, the chalcone form, and the quinonoidal form. In addition, at this high pH and 37 °C, anthocyanins degrade very quickly. For that reason, conditions were optimized to obtain the maximum amount of methylation product in the shortest time possible. Another limiting factor is the reduced amount of labeled AdoMet that can be used in radiosynthesis. Therefore, a high enzyme concentration (5000 units mL^{-1}) and high substrate concentration were used to maximize yield. High buffer capacity was also important to make sure that acid in the reagent solution, or the base to neutralize it, did not affect the final pH.

Solid phase extraction gave clean separation of anthocyanins from the other reagents including [*methyl*-^{3}H]AdoMet. Because of the low mass of product, it was possible to perform a preparative HPLC with an analytical column that separated malvidin-3-glucoside from the other anthocyanins. Initial preparative work was attempted with a LiChrospher RP-18 column. With this type of column, malvidin-3-glucoside eluted after the isomer, which inevitably caused contamination. A Luna C18-2 column inverted the order of elution, malvidin-3-glucoside being the first to elute (**Figure 2a**). Fractions containing [3′-*O*-*methyl*-^{3}H]malvidin-3-glucoside (**Figure 2b**) were combined to calculate specific activity based on absorbance and radioactivity.

On the basis of spectral quantification of the labeled product with a pure standard and total radioactivity measured by internal standard addition, the specific activity was 2.2 Ci mmol^{-1} for [3′-*O*-*methyl*-^{3}H]malvidin-3-glucoside, and 7.9 Ci mmol^{-1} was estimated for the isomer. The reduction in specific activity for [3′-*O*-*methyl*-^{3}H]malvidin-3-glucoside from 15 Ci mmol^{-1} in

the [*methyl*-^{3}H]Adomet starting material is mainly due to the presence of malvidin-3-glucoside in petunidin-3-glucoside starting material. In addition, minor contamination of the product may slightly lower the specific activity. The low specific activity of the isomer may be caused by a different molar absorptivity as no standard is available. The overall yield of [3′-*O*-*methyl*-^{3}H]malvidin-3-glucoside was 0.011 mCi indicating a 1.1% incorporation from the original 1 mCi of [*methyl*-^{3}H]AdoMet. For the isomer, the amount synthesized was 0.087 mCi, an 8.7% incorporation.

Attempts to recycle the [*methyl*-^{3}H]AdoMet that eluted with acidified water from the solid phase extraction to a follow-up reaction were not successful. On a related subject, an alternative to [*methyl*-^{3}H]AdoMet is [*methyl*-^{14}C]AdoMet, which would generate [3′-*O*-*methyl*-^{14}C]malvidin-3-glucoside, but conditions need to be optimized.

An important consideration is the rapid degradation of phenolic compounds in a dilute solution. It is advisable to do the enzymatic synthesis and purification right before the product is used. It is possible to store the sample after solid phase extraction. The best storage conditions observed include an acidic solution with a high percentage of methanol and high phenolic concentration (petunidin-3-glucoside) at 5 °C and excluding oxygen. However, the purified product cannot be stored due to breakdown, presumably from oxidation.

Therefore, [3′-*O*-*methyl*-^{3}H]malvidin-3-glucoside can be used to study the chemical fate of anthocyanins. An important advantage of the labeled material is that chemical transformation of anthocyanins into new products with no color, polymerization, or degradation products can still be traced.

ABBREVIATIONS USED

COMT, catechol-*O*-methyl transferase; AdoMet, *S*-adenosyl-L-methionine.

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